University of Alberta

Host-mediated regulation of a mobile genetic element

by

William Ryan Will

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Spring 2006

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-494-14062-3
Our file Notre référence
ISBN: 0-494-14062-3

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Transfer of the conjugative F plasmid of *Escherichia coli* is a tightly regulated process, as plasmid transfer and transfer (*tra*) gene expression occur only during exponential growth. The plasmid regulatory circuit consists of three proteins: TraJ, TraY, and TraM. TraJ, expressed from its own monocistronic operon, is the primary activator, up-regulating transcription of the 33 kb polycistronic *tra* operon, which encodes most of the proteins necessary for transfer. TraY, encoded by the first gene in the *tra* operon, further regulates *tra* operon expression and activates the transcription of *traM*, a monocistronic operon located upstream of *traJ*. TraM then represses its own expression. Results presented in this study indicate that the host nucleoid-associated protein, H-NS, is the central repressor in the *tra* regulatory circuit, silencing *tra* gene expression as donor cells enter stationary phase. *In vitro* studies demonstrate that H-NS binds to each of the *tra* promoters, and transcriptional analyses indicate that *tra* gene expression is de-repressed in *hns* mutant cells as they enter stationary phase. Furthermore, genetic studies indicate that TraJ, previously thought to be essential for transfer, is not essential in an *hns* mutant. Similar observations were also made for TraY. This suggests that the plasmid-encoded regulatory proteins act to disrupt H-NS-mediated silencing.

A regulatory role was also identified for the host RNA chaperone, Hfq. Genetic studies indicated that plasmid transfer was increased in stationary phase *hfq* mutant cultures. Stationary phase TraM and TraJ levels are increased in *hfq* mutant cultures, and the stability of transcripts containing the *traJ* 5' untranslated region (UTR) is increased in *hfq* strains. Biochemical studies indicate that Hfq binds the 5' UTR of *traJ* mRNA,

suggesting that Hfq destabilizes transcripts containing this region, repressing plasmid transfer.

A screen of the other major nucleoid-associated proteins, Fis, IHF, HU, and StpA, was also performed. Of these, IHF, which is a critical component of the F relaxosome, had the most significant effect, positively regulating all three regulatory genes. These results indicate that *tra* gene regulation is a complex process, receiving regulatory input from several host factors.

Acknowledgements

While many people helped me during the course of this thesis, I should start by thanking my supervisor, Dr. Laura Frost. Laura not only took a chance and offered me a position in her lab when I was still an inexperienced scientist and researcher, but allowed me the freedom to develop my own ideas and projects, which I think has helped me a great deal. I would also like to acknowledge the support of my committee, Dr. Diane Taylor, Dr. Brenda Leskiw, and Dr. Tracy Raivio during Dr. Leskiw's sabbatical. I particularly appreciated Dr. Leskiw's support, as her door was always open to provide advice or reagents.

I would also like to thank the many members of the Leskiw lab, past and present. For most of my degree, Kent Gislason, Dawn Bignell, Kim Colvin, and the rest of the gang worked next door and were always willing to share scientific advice, jokes, or at the very least, tolerate the racket coming from my side of the lab, an act of kindness and understanding which did not go unnoticed.

Dr. Bill Page and his laboratory also deserve thanks for allowing me to perform much of the Northern analyses presented in this thesis, in their lab. James Sandercock was particularly helpful, always willing to provide discussion, scientific and otherwise, and no doubt saved me a significant amount of time and grief completing this work.

This thesis was also helped greatly by assistance and advice from various members of nucleoid-associated protein community, who provided a wide array of strains, constructs, and proteins. Dr. Sylvie Rimsky at the Université Paris XI was particularly helpful in providing pure H-NS, as well as directing me to the appropriate person whenever I needed additional assistance.

I have also been helped a great deal by the many people to pass through the Frost lab over the years. However, three people in particular deserve special thanks for the impact they had during my time in the lab. Jan Manchak, beyond being the best technician that I have ever had the pleasure of working with, and an exceptional scientist, was the lab mom, and made sure everything went harmoniously in the lab. She was always willing to offer her time and advice, teaching me most of the basic techniques I learned in my early days in the lab. She also helped to keep lab life enjoyable, whether it meant joining in on jokes and songs or coffee. Dr. Mike Gubbins took me under his wing and was my mentor, or perhaps dementor, early on. Aside from sharing my particularly twisted sense of humor, Mike was always willing to listen to and encourage my scientific ideas, and set a high standard for me to strive for in the lab. Dr. Samantha Holland helped pick up where Mike left off. She was always willing to provide scientific advice, and was a great friend and drinking/coffee/hockey partner, making the later stages of my degree much more enjoyable than they might otherwise have been.

Lastly, I would like to thank my family. My parents encouraged the scientific pursuits of all their children from a very early age, and my siblings provided a healthy amount of intellectual competition as we grew up. They supported me, even through the rough patches, and for that, I am grateful.

Table of contents

Chapter 1: General Introduction

1.1	DNA supercoiling as a global regulator	2
1.2	Fis, a dynamic nucleoid-associated protein	10
1.3	IHF, the nucleoid associated-protein	13
1.4	HU, the nucleoid associated-protein	18
1.5	H-NS, the universal repressor	20
1.6	Hfq, the global RNA chaperone	33
1.7	Nucleoid-associated proteins and horizontal gene transfer	41
1.8	A brief overview of the F plasmid	43
1.9	The F plasmid relaxosome	46
1.10	F plasmid *tra* gene regulation	47
1.11	Summary and research objectives	51

Chapter 2: Materials and Methods

2.1	Bacterial strains and growth conditions	55
2.2	Recombinant DNA techniques and plasmid construction	55
2.3	Mating assays	62
2.4	Curvature prediction	63
2.5	Competitive H-NS electrophoretic mobility shift assays	63
2.6	DNase I footprinting assays	64
2.7	Immunoblot analysis	65
2.8	*In vitro* transcription	66

2.9 Northern blot analysis 67

2.10 Primer extension analysis 69

2.11 β-galactosidase assays 70

2.12 Overexpression and purification of Hfq 71

2.13 Hfq electrophoretic mobility shift assays 72

2.14 Isoelectric focusing analysis 72

2.15 Fis and IHF electrophoretic mobility shift assays 73

Chapter 3: The role of H-NS in silencing F transfer gene expression during entry into stationary phase

3.1 Introduction 76

3.2 Results

3.2.1 Transfer efficiency and growth is altered in an *hns* mutant 80

3.2.2 DNA curvature prediction at *traM* and *traJ* 83

3.2.3 H-NS binds to the P_M and P_J regions 86

3.2.4 DNase I footprinting of the P_J promoter 87

3.2.5 TraJ and TraM levels are increased in *hns* cells 87

3.2.6 Analysis of *traM* and *traJ* mRNA levels in *hns* cells 92

3.2.7 β-galactosidase fusion analysis of *traM* and *traJ* expression 100

3.3 Discussion 102

Chapter 4: Characterization of the opposing roles of H-NS, TraJ, and TraY in the transcriptional regulation of F plasmid *tra* gene expression

4.1 Introduction 109

4.2 Results

4.2.1 H-NS binds to intrinsically curved DNA at P_Y 113

4.2.2 H-NS down-regulates transcription from P_Y 116

4.2.3 TraJ is unnecessary for F plasmid transfer from an *hns* host 122

4.2.4 TraJ opposes H-NS in the regulation of *tra* gene expression 123

4.2.5 TraY opposes H-NS-mediated repression of *tra* gene expression 129

4.3 Discussion 129

Chapter 5: Hfq is a regulator of F plasmid TraJ and TraM synthesis

5.1 Introduction 138

5.2 Results

5.2.1 Hfq represses plasmid transfer as donor cell cultures enter stationary phase 144

5.2.2 Stationary phase TraJ and TraM levels are increased in an *hfq* host 145

5.2.3 Hfq-mediated growth rate-dependent regulation of TraJ levels 154

5.2.4 Hfq binds the *traM-traJ* intergenic UTR 154

5.2.5 Plasmid transcriptional profiles are altered in an *hfq* mutant host 157

5.2.6 Transcript stability is increased in *hfq* mutant host cultures 162

5.3 Discussion 166

Chapter 6: The effect of nucleoid-associated proteins on F plasmid transfer: a general activational role for IHF

6.1 Introduction 171

6.2 Results

6.2.1 Mating efficiency analysis of nucleoid-associated protein mutants 175

6.2.2 Immunoblot analysis of F plasmid *tra* regulatory proteins in nucleoid-associated protein mutant host strains 178

6.2.3 Transcriptional analysis of nucleoid-associated protein mutants 181

6.3 Discussion 184

Chapter 7: General Discussion

7.1 H-NS: the master repressor of *tra* gene expression 188

7.2 *tra* silencing is context-dependent 188

7.3 A new role for TraJ: H-NS antagonist 194

7.4 IHF activates plasmid transfer on multiple levels 196

7.5 Hfq stimulates degradation of transcripts containing the 5' UTR 197

7.6 Re-programming the host: the role of plasmid-encoded homologs 198

7.7 Fine-tuning the signal: the role of additional regulatory factors 199

7.8 Evolution of a plasmid regulatory circuit 200

7.9 Future directions 202

Chapter 8: Appendix I – F plasmid transfer is sensitive to acetate metabolism

8.1 Introduction 206

8.2 Results and Discussion

8.2.1 Mating efficiency is sensitive to acP accumulation 209

8.2.2 TraY levels are sensitive to acP accumulation 210

Chapter 9: Appendix II – TraJ is post-translationally modified

9.1 Introduction 216

9.2 Results and Discussion 216

Chapter 10: Appendix III – DNA binding by Fis and IHF at P_Y

10.1 Introduction 221

10.2 Results and Discussion 221

List of figures

Figure 1.1	The effect of supercoiling on the DNA duplex	5
Figure 1.2	Chromosomal condensation and supercoiling promotes long-distance interactions	9
Figure 1.3	IHF-DNA and HU-DNA co-crystal structures	15
Figure 1.4	IHF-mediated regulation of *ilv*P_G	18
Figure 1.5	A model for the function of HU in the Gal repressosome	22
Figure 1.6	H-NS oligomerization and strand-bridging	26
Figure 1.7	Two models for H-NS-mediated repression	29
Figure 1.8	A model for transcriptional regulation of *virF*	32
Figure 1.9	The structure of *Staphylococcus aureus* Hfq	36
Figure 1.10	Mechanisms of DsrA-mediated regulation	39
Figure 1.11	The bacterial conjugation cycle	45
Figure 1.12	The basic *tra* regulatory circuit	49
Figure 3.1	A model for the regulatory circuit	78
Figure 3.2	F plasmid transfer is prolonged in an *hns* mutant	82
Figure 3.3	The *traM* and *traJ* promoter regions contain regions of predicted curvature	85
Figure 3.4	H-NS binds preferentially to the *traJ* and *traM* promoter regions	89
Figure 3.5	Analysis of H-NS binding at the *traJ* promoter by DNase I footprinting	91
Figure 3.6	Immunoblot analysis of TraM, TraJ, and TraY in an *hns* mutant	94
Figure 3.7	Northern analysis of transcript levels for both *traM* and *traJ*	97

Figure 3.8	Primer extension analysis of P_M and P_J	99
Figure 4.1	The F plasmid regulatory circuit	112
Figure 4.2	The P_Y region contains significant intrinsic curvature	115
Figure 4.3	H-NS binds preferentially to the P_y region	118
Figure 4.4	H-NS represses *traY* transcription as donor cells approach stationary phase	121
Figure 4.5	Immunoblot analysis of TraM and TraY from mutant plasmids in wild-type and *hns* mutant donor cells	126
Figure 4.6	TraJ opposes H-NS mediated repression of *tra* gene expression	128
Figure 4.7	TraY opposes H-NS-mediated repression of *tra* gene expression	131
Figure 5.1	The F plasmid regulatory circuit is composed of three major plasmid-encoded regulatory proteins: TraJ, TraY, and TraM	140
Figure 5.2	The secondary structure of *traJ*	143
Figure 5.3	F plasmid transfer is increased in stationary phase in *hfq1*::Ω (AM111) host cells	147
Figure 5.4	Stationary phase TraJ and TraM levels are increased in *hfq1*::Ω (AM111) host cells	151
Figure 5.5	Hfq is not necessary for FinOP-mediated repression of TraJ synthesis	153
Figure 5.6	Hfq-mediated repression is growth rate-dependent	156
Figure 5.7	Hfq specifically binds the *traJ* 5' UTR	159
Figure 5.8	*traJ* and *traM* transcript profiles are altered in an *hfq1*::Ω (AM111) host strain	161

Figure 5.9	*traJ* and *traM* transcript stability is altered in an *hfq1*::Ω host strain	165
Figure 6.1	The effect of nucleoid-associated proteins on F plasmid transfer	177
Figure 6.2	The effect of nucleoid-associated proteins on F plasmid Tra regulatory proteins	180
Figure 6.3	F plasmid regulatory transcript levels in nucleoid-associated Protein mutant host strains	183
Figure 7.1	Regulation of F plasmid transfer	190
Figure 7.2	A model for three-dimensional nucleation and cooperative silencing	193
Figure 8.1	The Pta-AckA pathway	208
Figure 8.2	TraY synthesis is sensitive to acP accumulation	213
Figure 9.1	TraJ is post-translationally modified	219
Figure 10.1	Predicted IHF and Fis binding sites upstream of P_Y	224
Figure 10.2	IHF and Fis bind the P_Y region	226

List of tables

Table 2.1	Bacterial strains used in this study	56
Table 2.2	Plasmids used in this study	59
Table 2.3	Oligonucleotides used in this study	61
Table 4.1	Plasmid transfer of F*lac traJ90* is partially restored in an *hns* host strain	124
Table 5.1	Hfq is not required for FinOP-mediated repression of F plasmid transfer	148
Table 8.1	F plasmid mating efficiency is sensitive to acP accumulation	211

List of Abbreviations

α	alpha
A	adenine
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
Ace	acetate
Ack	acetate kinase
acP	acetyl phosphate
ADP	adenosine diphosphate
Amp/Ap	ampicillin
ATP	adenosine triphosphate
β	beta
bla	beta-lactamase
bp	base pair
C	cytosine
cAMP	cyclic adenosine monophosphate
Cm	chloramphenicol
cpm	counts per minute
Cpx	conjugative pilus expression
CRP	cAMP receptor protein
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
F	Fertility sex factor
Fin	fertility inhibition
Fis	Factor for inversion stimulation
γ	gamma
g	gravity
G	guanine
hbs	HU binding site
Hfq	Host factor for phage Qβ replication
H-NS	Histone-like nucleoid structuring
hr	hour
HU	Heat unstable
IEF	Isoelectric focusing

IHF	Integration host factor
Inc	incompatibility
IPTG	Isopropyl β-D-thiogalactopyranoside
IS	insertion sequence
kan/Km	kanamycin
kb	kilobase
K_d	equilibrium dissociation constant
kDa	kilodalton
Lac	lactose
LB	Luria-Bertani
LCR	locus control region
LEE	locus of enterocyte effacement
Lk	linking number
Lrp	leucine responsive protein
M	molar
mA	milliampere
ml	milliliter
mg	milligram
mM	millimolar
MOPS	3-(N-Morpholino)propane-sulfonic acid
mRNA	messenger RNA
MU	Miller units
Ni^{2+}-NTA	nickel-nitrilotriacetic acid
nic	nick site
nM	nanomolar
NTP	nucleotide triphosphate
OD_{600}	optical density at 600 nm
ORF	open reading frame
oriT	origin of transfer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
P_{finP}	promoter for *finP*
pmol	picomole
P_J	promoter for *traJ*
pM	picomolar
P_M	promoter for *traM*
Pta	phosphotransacetylase
P_Y	promoter for *traY*
R	resistant

RBS	ribosome binding site
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
sbm	site of binding for TraM
sby	site of binding for TraY
SDS	sodium dodecyl sulfate
SIDD	stress-induced duplex destabilization
SL	stem-loop
Spc	spectinomycin
sRNA	small RNA
T	thymine
Tc	tetracycline
TCST	two component signal transduction
Tn	transposon
Topo	topoisomerase
Tra	transfer
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
T_{traM}	*traM* terminator
Tw	twist
U	unit
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
Vir	virulence
Wr	writhe
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromolar
°C	degrees Celsius

Chapter 1: General Introduction

Bacteria can grow in a wide array of varied environments, each of which can impose a unique set of physiological and metabolic conditions upon the cell. In order to cope with this fact, bacterial cells are forced to develop an elaborate global regulatory circuitry which is capable of fine-tuning gene expression patterns so that unwanted or unnecessary gene expression is limited as much as possible to avoid energetic waste. This circuitry is formed by a series of overlapping regulatory hierarchies. The first and most general level of control is at the level of chromosomal structure. Chromosomal structure, controlled by a large group of architectural proteins, influences the accessibility of DNA and sensitivity of gene expression to more specific regulatory inputs. The second level of control consists of large groups of genes which respond to specific environmental and metabolic signals, referred to as stimulons or regulons. Gene expression is fine-tuned by the third level of control, which consists of operon-specific signals, working in conjunction with the more general regulatory elements.

1.1 DNA supercoiling as a global regulator

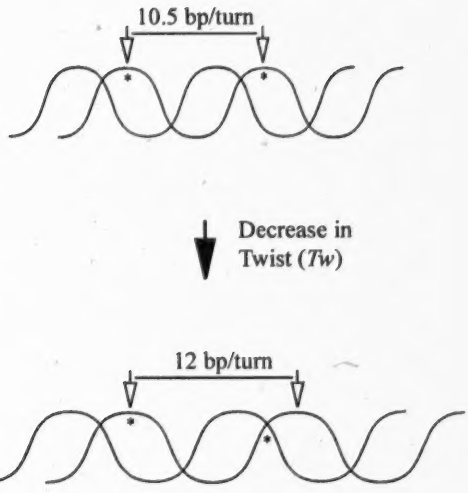
The critical factor that determines gene expression patterns is the level of energy available in the cell, necessary to support all cellular functions. General chromosome structure is sensitized to this via DNA gyrase and DNA supercoiling. Bacterial DNA generally exists in an underwound, or negatively supercoiled state (Drlica, 1992), and this state is maintained by a set of enzymes referred to as topoisomerases, which are responsible for nicking one or two strands and adding or removing negative supercoils as appropriate. While several topoisomerases exist, two are critical to maintaining the overall topological state of the bacterial chromosome in *Escherichia coli*. Topoisomerase (Topo) I is responsible for removing excess negative supercoils, nicking a single strand of

the DNA duplex and allowing it to cross over the other strand of the duplex (Zechiedrich *et al.*, 2000; Champoux, 2001). This results in the relaxation of the chromosome. The activity of Topo I is ATP-independent, requiring only negative supercoiling to drive its activity. The second critical enzyme is DNA gyrase (topoisomerase II), which introduces negative supercoils to the DNA by way of an ATP-dependent reaction, nicking both strands and passing them over each other (Champoux, 2001). However, despite its requirement of ATP for activity, DNA gyrase does not respond directly to ATP levels, but rather [ATP]/[ADP] ratios which are defined by cellular free energy (Higgins *et al.*, 1988; Hsieh *et al.*, 1991). Hence, chromosomal supercoiling is tied directly to the metabolic state.

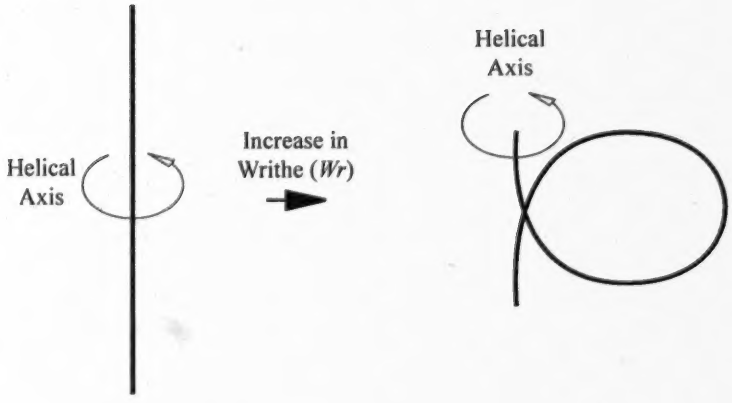
The overall topology of a chromosome or particular DNA molecule is described by the linking number (*Lk*), which is determined by the sum of two variables, twist (*Tw*), and writhe (*Wr*) (Fig 1.1) (White, 1969). Twist refers to the number of turns around the helical axis of a particular DNA duplex, whereas the writhe number refers to the number of turns made by that duplex around a superhelical axis, forming toroidal loops. The linking number is constant within a covalently closed molecule. Hence, fluctuations in *Tw* must be countered by the opposite change in *Wr*, or vice versa. The superhelicity of a DNA molecule is determined by comparing the current or changed linking number (ΔLk) to the linking number of a molecule in its native, relaxed state (Lk_o). The relative topology or superhelical density of a molecule can then be indicated by $\sigma = \Delta Lk / Lk_o$. As the bacterial chromosome is generally underwound in physiological conditions, the superhelical density, σ, is negative.

Figure 1.1 The effect of supercoiling on the DNA duplex. DNA supercoiling is indicated by two variables: twist (*Tw*) (A), and writhe (*Wr*) (B). Under normal physiological conditions in a bacterial cell, chromosomal DNA exhibits a *Tw*, or the rate of turn around the helical axis, of approximately 10.5 bp per helical turn (A). Changes in *Tw* will result in residues moving out of phase, altering interaction surfaces for protein-binding. In a covalently closed molecule, unless the duplex is nicked to allow changes in supercoiling, a change in *Tw*, must be met by an equal and opposite change in *Wr*, resulting in coiling of the duplex around a superhelical axis.

A)


10.5 bp/turn
Decrease in
Twist (*Tw*)
12 bp/turn

B)


Helical
Axis
Increase in
Writhe (*Wr*)
Helical
Axis

A bacterial chromosome during exponential growth is generally maintained at $\sigma \approx -0.05$ (Kusano *et al.*, 1996). However, the degree of superhelicity can fluctuate in response to both physiological and environmental stimuli, falling to levels of -0.03 during stationary phase, or increasing to -0.09 during periods of environmental stress, such as osmotic shock, which causes a transient increase in the intracellular [ATP]/[ADP] ratio (Higgins *et al.*, 1988).

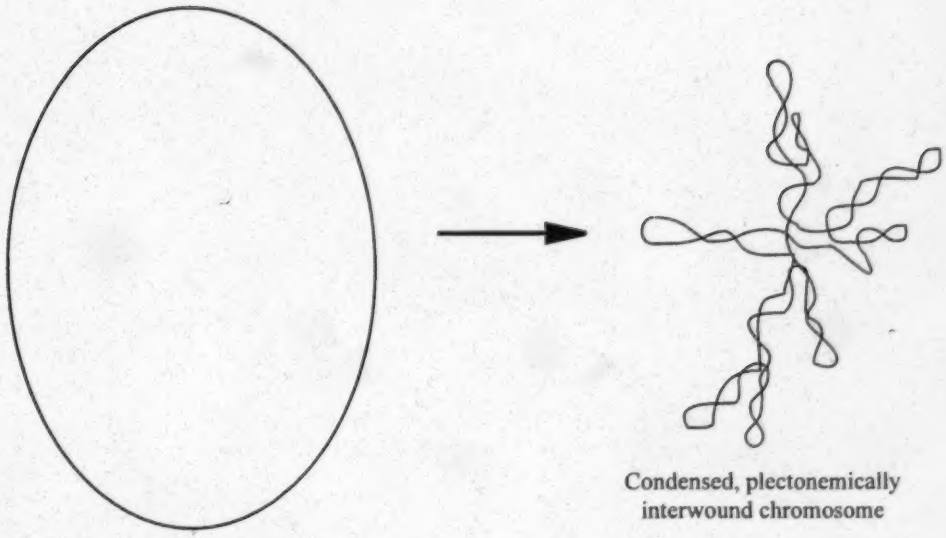
Fluctuations in chromosomal topology can alter gene expression a number of ways. One clear example is the alteration of helical phasing of residues in the DNA duplex via fluctuations in *Tw*. While a series of residues might lie on the same helical face at a standard helical rate of turn (≈10.5 bp per turn), altering the *Tw* of that region will shift the same residues out of phase (Fig 1.1A). If the positioning of the residues is critical for the binding of a regulatory factor, binding activity will likely be altered. The clearest example of this is the effect of fluctuations in the -10 to -35 spacer at bacterial promoters on RNA polymerase binding and hence, transcriptional initiation (Wang and Syvanen, 1992). The optimum spacing under standard physiological conditions is 17 base pairs, and deviations from this standard spacer length can result in promoters extremely sensitive to fluctuations in helical twist (Steck *et al.*, 1993). Dynamic supercoiling can also drive structural changes in the chromosome. These can include the formation of cruciform structures at inverted repeats (Dayn *et al.*, 1992), transitions from B-form to Z-form DNA (Aboul-ela *et al.*, 1992), and regions of local denaturation, referred to as stress-induced duplex destabilization (SIDD) (Kowalski *et al.*, 1988). SIDDs promote open complex formation and hence, transcriptional initiation (Drew *et al.*, 1985). These structural distortions also serve to relax the overall superhelicity of

local DNA as well as modulate affinity of DNA-binding proteins for such sites. Conversely, protein binding at these sites can, in some cases, force the DNA to return to a B-form duplex, increasing local superhelicity. Alternatively, structural reversions caused by protein binding can transmit their superhelical energy to the nearest site capable of distorting to dissipate the trapped energy. Sequences capable of forming these structural distortions can effectively compete with each other for the superhelical energy. This competition can be modulated by bound proteins which channel the transmission of helical energy to the appropriate site. Supercoiling also plays a key role in the facilitation of rapid movement of DNA-binding proteins to their most preferential targets (Fig 1.2). Supercoiled DNA is plectonemically interwound, reflective of its writhe number. As a supercoiled molecule reptates around itself in an attempt to dissipate trapped superhelical energy, two one-dimensionally distant protein-binding sites can become quite close, three-dimensionally, to each other. Because of this interwinding, the three-dimensional supercoiled distance between two distant binding sites cannot be any greater than the one dimensional distance in a relaxed molecule (Huang *et al.*, 2001). This characteristic facilitates the dissociation and three-dimensional diffusion of bound proteins to their preferential targets much more rapidly than simple one-dimensional processive diffusion can allow, provided that a protein is capable of movement other than simple one-dimensional diffusion (Gowers and Halford, 2003; Kampmann, 2005).

As much as 50% of the supercoiling within a chromosome is constrained by the binding of proteins, including replication factors, RNA polymerase, and the architectural nucleoid-associated proteins, such as HU, IHF, H-NS, and Fis (Rouviere-Yaniv *et al.*, 1979; Pettijohn and Pfenninger, 1980; Tupper *et al.*, 1994). Beyond simply constraining

Figure 1.2 Chromosomal condensation and supercoiling promotes long-distance interactions. Whereas a relaxed, decondensed chromosome occupies a large space, a condensed, plectonemically supercoiled chromosome is relatively compact (A). The interwound chromosome can bring one-dimensionally distant sites into close three-dimensional contact, which can facilitate rapid diffusion of DNA-binding proteins, as well as promote interactions between distant sites on the chromosome (B).

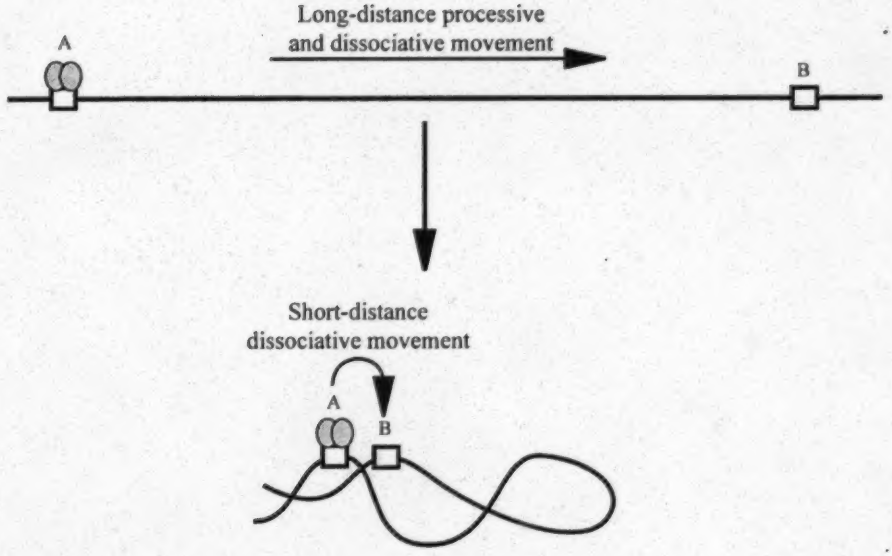
A)



Decondensed, relaxed chromosome

Condensed, plectonemically interwound chromosome

B)



supercoils, some proteins, such as RNA polymerase, separate the duplex strands and move processively along the DNA can also generate fields of positive and negative supercoiling ahead of and behind the protein, respectively. This is referred to as the two-domain model (Liu and Wang, 1987; Wu *et al.*, 1988). Unconstrained, these waves of supercoiling can diffuse through the chromosome, eventually colliding and canceling each other out. However, chromosomal DNA is usually well constrained. As a result, transcription from either convergent or divergent promoters can result in domains of highly underwound or overwound DNA, until cellular topoisomerases are able to restore the DNA to its typical topological state, or an opposing field of supercoiling cancels the other out.

As mentioned above, bacterial histone-like or nucleoid-associated proteins play an important role in controlling the structure of the bacterial chromosome by constraining supercoiling. However, their roles are more complex than simply providing a protein scaffolding for the chromosome. They function as specific regulators of all aspects of DNA metabolism, including replication, recombination, and transcription (Beloin *et al.*, 2003). While there are many nucleoid-associated proteins in *Escherichia coli*, four appear to play the most significant roles in the cell, particularly during active growth: Fis (Factor for inversion stimulation), IHF (Integration host factor), HU (Heat unstable), and H-NS (Histone-like nucleoid-structuring).

1.2 Fis, a dynamic nucleoid-associated protein

Originally characterized as an enhancer of Hin, Cin, and Gin recombinases (Johnson and Simon, 1985; Kahmann *et al.*, 1985; Haffter and Bickle, 1987; Johnson *et al.*, 1988), Fis has been shown to be involved in many other cellular functions. Fis has

roles in regulating initiation of DNA replication at *oriC* (Filutowicz *et al.*, 1992), λ phage excision and integration (Ball and Johnson, 1991b), and transcriptional regulation (Finkel and Johnson, 1992). Fis is a particularly common regulatory factor in growth-phase dependent gene control, due in part to its dynamic expression pattern. Fis is synthesized only briefly in early exponential phase, peaking at approximately 60,000 molecules per cell, in response to the nutritional quality of the growth medium (Ball *et al.*, 1992; Ali Azam *et al.*, 1999). By stationary phase, intracellular Fis has become nearly undetectable. This expression pattern is due to a complex regulatory circuit, influenced by the stringent response (Ninnemann *et al.*, 1992), intracellular NTP pools (Walker and Osuna, 2002; Walker *et al.*, 2004), IHF (Pratt *et al.*, 1997), CRP (Nasser *et al.*, 2001), DNA supercoiling (Schneider *et al.*, 2000), and autoregulation (Walker *et al.*, 1999). Fis is also regulated post-transcriptionally by the translation factor, BipA, which is thought to destabilize strong interactions between the ribosome and the 5' untranslated region (UTR) of *fis* mRNA, promoting translation (Owens *et al.*, 2004).

DNA supercoiling appears to be critical in *fis* regulaton. Studies have demonstrated that transcription of *fis* is activated by high supercoiling at its promoter (Schneider *et al.*, 2000). As DNA gyrase activity is dependent on cellular free energy, so is Fis synthesis. Conversely, Fis represses transcription of *gyrA* and *gyrB*, which encode DNA gyrase subunits (Schneider *et al.*, 1999). As a result, a negative feedback loop exists between Fis levels and DNA supercoiling. This loop is particularly significant when one considers that Fis regulates the expression of several other nucleoid-associated and global regulatory proteins (see below).

Like many of the nucleoid-associated proteins, Fis DNA binding activity is not highly sequence-specific. Although a weak binding consensus logo exists (Hengen *et al.*, 1997), Fis binding appears to be more structure-specific than sequence-specific. A consensus Fis binding site consists of highly conserved G and C residues at the -7 and +7 positions, respectively, centered around an AT-rich bend center approximately 7 bp in length. The bend appears to be well conserved, but its sequence is not.

Fis is a central transcriptional activator for the expression of stable RNAs, including rRNA and tRNA, which are necessary for protein synthesis during active growth (Zhang and Bremer, 1996; Hirvonen *et al.*, 2001; Schneider *et al.*, 2003). The peak in intracellular Fis levels in early exponential phase allows for the rapid activation of tRNA and rRNA synthesis necessary during growth. However, Fis is also associated with the regulation of many other genes including those encoding regulatory factors, virulence factors, and proteins associated with biofilm formation (Dorman and Deighan, 2003).

The general mode of action for Fis appears to involve modulation of supercoiling (Muskhelishvili and Travers, 2003). Beyond its role as a regulator of DNA gyrase synthesis (Schneider *et al.*, 1999), Fis appears to promote branching of supercoiled bacterial DNA (Schneider *et al.*, 2001). Each branch results in the formation of an additional apical loop in the chromosome. Segments of curved DNA, like those found in UASs (upstream activating sequences) upstream of the stable RNA promoters, will be preferentially located at the apices (Laundon and Griffith, 1988; Rippe *et al.*, 1995). As RNA polymerase preferentially associates with the apical loops of supercoiled DNA, due to a requirement for DNA wrapping around the polymerase during transcriptional

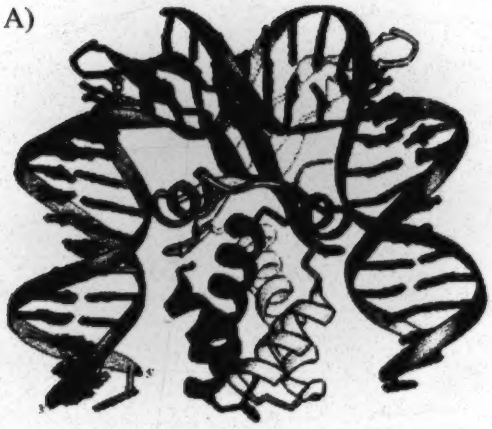
initiation (Coulombe and Burton, 1999), apical loop formation, and hence branch formation, effectively increases the number of high affinity RNA polymerase targets. As a result, promoters located at or near these apical loops will be preferentially activated. The final result of Fis activity is an increase in the transcriptional activity of the subset of promoters located at the apical loops, due to increased RNA polymerase affinity.

1.3 IHF, the nucleoid-associated protein

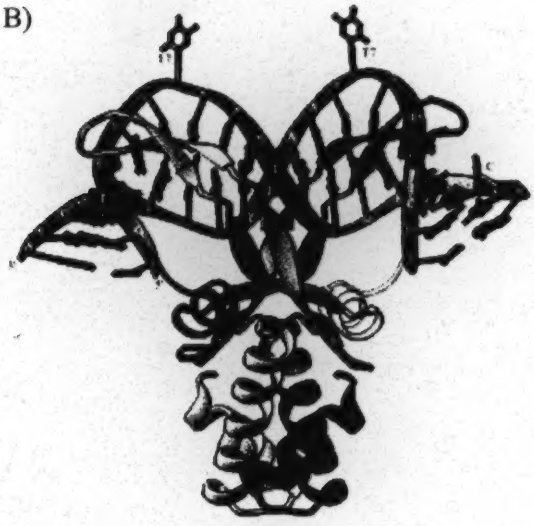
Originally identified for its role in λ integrative recombination (Miller and Friedman, 1980), IHF is involved in all aspects of DNA metabolism, including recombination, initiation of replication, and gene regulation (Friedman, 1988). Unlike Fis, IHF is heterodimeric, encoded by two genes, *himA*, and *himD*, or *hip*, located at 38 and 25 min, respectively, on the chromosome (Miller and Friedman, 1980; Kikuchi *et al.*, 1985). Also unlike Fis, IHF binding is relatively sequence-specific, recognizing the moderately conserved consensus sequence: YAANNNNTTGATW (where W = A or T, and Y = T or C) (Goodrich *et al.*, 1990). DNA bound by IHF is bent over 160°, wrapping around the protein, with much of the bending occurring at two sharp kinks in the duplex, due to the intercalation of a proline residue from the protein arms (Fig. 1.3A) (Rice *et al.*, 1996). This structure has multiple functional implications. First, it allows IHF to act as a looper molecule, promoting the formation of DNA loops, and allowing direct interaction between distant sites, as in the case of λ integration, where it brings integrase binding sites into close proximity with bacterial and phage attachment sites (Moitoso de Vargas *et al.*, 1989). Bound IHF has also been shown to modulate supercoiling by preventing normal translocation of superhelical energy necessary for SIDD formation from B duplex DNA near the IHF binding site, as demonstrated for the *ilvGMEDA* operon

Figure 1.3 IHF-DNA and HU-DNA co-crystal structures. The structure of IHF bound to a nicked DNA fragment containing the H' binding site from bacteriophage λ (A). The α (*himA*) subunit is in white, and the β (*himD*/*hip*) subunit is indicated in pink. Intercalating proline residues are visible at the tips of the binding arms, indicated in yellow. The consensus binding sequence is indicated in green. Adapted by permission from Elsevier: *Cell.* 87: Crystal structure of an IHF-DNA complex: a protein-induced DNA U-turn. Rice *et al.*, 1295-1306 (1996). The structure of the HU α2 homodimer (B). Again, intercalating proline residues, indicated in yellow, are visible at the tips of the binding arms. Adapted by permission from Macmillan Publishers Ltd: *EMBO J.*, 22: Flexible DNA bending in HU-DNA cocrystal structures. Swinger *et al.*, 3749-3760 (2003).

A)



B)



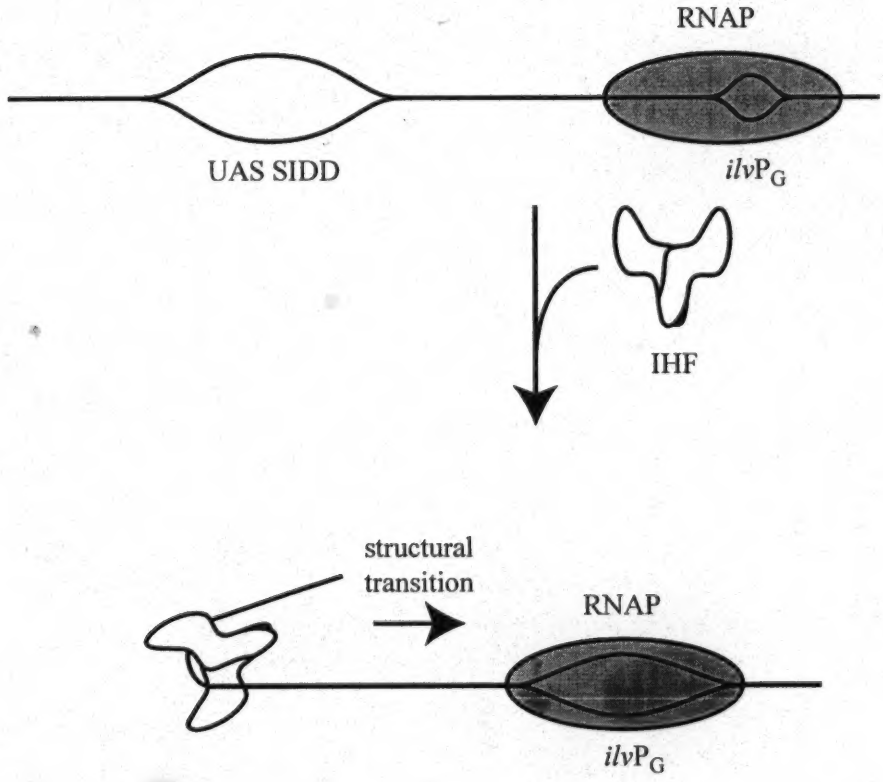
(Parekh *et al.*, 1996; Sheridan *et al.*, 1998; Sheridan *et al.*, 1999). As a result, the trapped superhelical energy is translocated to another region within the domain, where it is dissipated by SIDD formation or other means. This channeling often plays a role in promoting open complex formation, as in the case of the *ilvGMEDA* operon, where superhelical energy is transmitted to the -10 box of the promoter, resulting in SIDD formation (Fig. 1.4).

The expression profile of IHF is also quite different from that of Fis. Intracellular IHF levels are lowest in early exponential phase, at approximately 12,000 monomers per cell, but peak in early stationary phase, at approximately 55,000 monomers per cell (Ditto *et al.*, 1994; Ali Azam *et al.*, 1999). By late stationary phase, IHF levels have decreased to approximately 27,000 monomers per cell. Based on this profile, some have suggested that IHF plays an important role in organizing the bacterial nucleoid during the transition between exponential and stationary phase (Ali Azam *et al.*, 1999), however the fluctuation in protein levels is not as significant as for Fis. This expression profile allows IHF to regulate a wide array of genes, including those involved with biosynthesis, transport, cell structure, metabolism, environmental response, and regulation (Arfin *et al.*, 2000).

1.4 HU, the nucleoid-associated protein

HU is a small, 18 kDa dimeric protein, related to IHF, that is encoded by two separate genes: *hupA* (Kano *et al.*, 1985), which encodes the HUα subunit, and *hupB* (Kano *et al.*, 1986), which encodes the HUβ subunit. However, unlike IHF, HU is not solely heterodimeric. The α2 homodimer is the predominant species in exponential phase, while the αβ heterodimer becomes more common in stationary phase, with low

Figure 1.4 IHF-mediated regulation of *ilv*P_G. Superhelical energy promotes SIDD formation at the UAS upstream of *ilv*P_G. IHF binding at the UAS prevents SIDD formation and channels the excess superhelical energy to the -10 region of *ilv*P_G. This channeling promotes strand separation at the -10 region and increases open complex formation promoting transcriptional initiation by the RNA polymerase (indicated by the gray oval).


RNAP
UAS SIDD
$ilvP_G$
IHF
structural
transition
RNAP
$ilvP_G$

levels of the β2 homodimer also becoming detectable (Claret and Rouviere-Yaniv, 1997). The general expression profile is similar to that of IHF, with intracellular levels peaking at 30,000 dimers per cell during the transition between exponential and stationary phase, then dropping to approximately 10,000 dimers per cell in stationary phase (Ali Azam *et al.*, 1999).

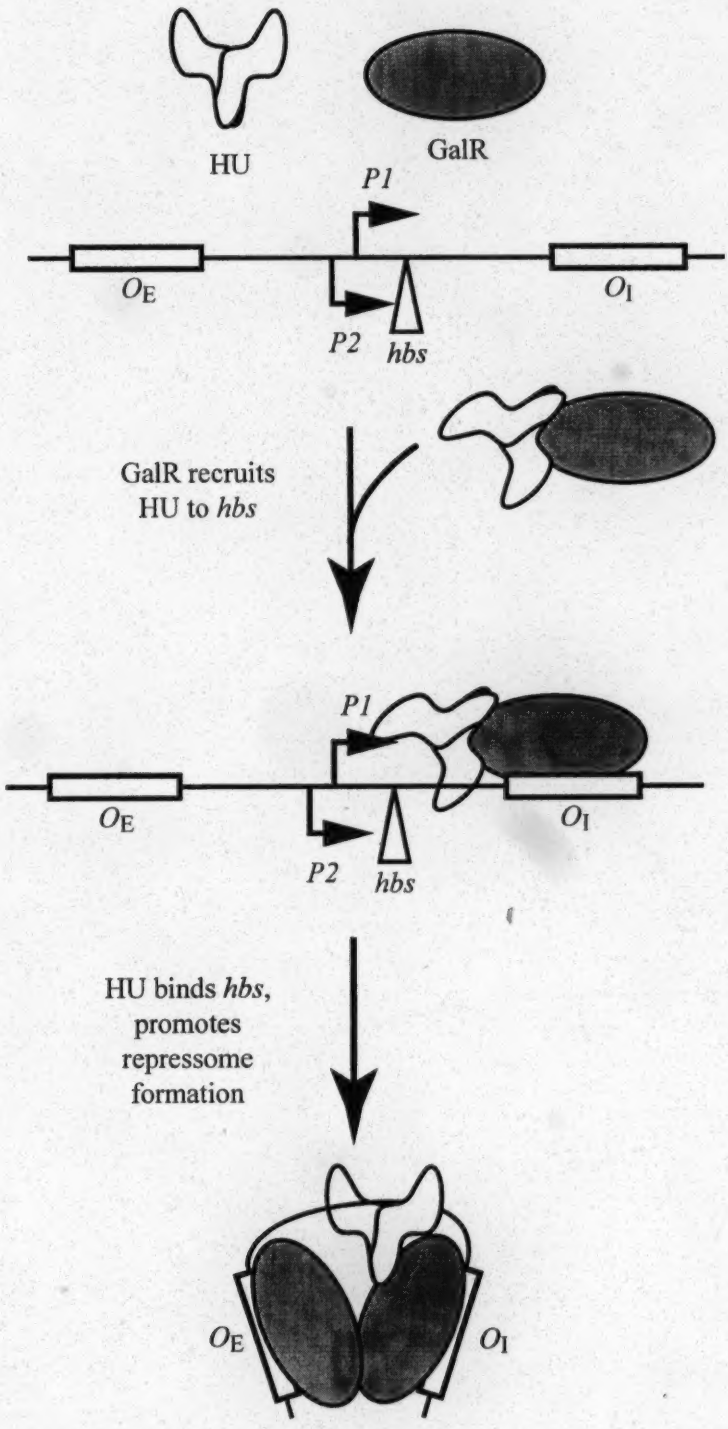
There are differences in the activities of the HUα and HUβ subunits of HU. Biochemical analysis has demonstrated that whereas the heterodimer and the α2 homodimer preferentially bind cruciform, kinked, and nicked or gapped linear DNA with high affinity under stringent conditions, the β2 homodimer is unable to recognize the nicked or gapped linear DNA, although it still binds cruciform DNA with high affinity (Pinson *et al.*, 1999). Whether or not this difference in target site preference has a significant function *in vivo* is unknown. However, given the transition in HU subunit composition in early stationary phase, it may play a role in structural changes in the nucleoid at this time. Similar to IHF, bound HU induces bending in the DNA duplex by way of proline intercalation (Swinger *et al.*, 2003). However, the overall bend angle stabilized by HU is not as significant (105°-140°) as in the IHF-DNA complex, and the two major kinks or bend angles in the HU-DNA complex are not coplanar (Fig 1.3B). Bound HU also appears to stabilize underwound, negatively supercoiled DNA, as the average twist in an HU-DNA complex is 31°/bp, less than the average of 34°/bp observed in the IHF complex, and 34.3°/bp in relaxed B-form DNA (Swinger *et al.*, 2003). Furthermore, the dihedral angle of the HU-DNA complex (40°-73°) suggests that HU is capable of introducing negative writhe into the bound duplex (Swinger *et al.*, 2003).

Like the other major nucleoid-associated proteins, HU has roles in replication, recombination, and repair, a function reflected by its preference for gapped or nicked DNA (Skarstad *et al.*, 1990; Dri *et al.*, 1992; Li and Waters, 1998; Hashimoto *et al.*, 2003). HU also appears to be involved in gene regulation; however, mechanistic understanding of how it does this is limited. The best studied system is the Gal repressosome, which regulates transcription of the *gal* operon in *E. coli* (Fig. 1.5). In this system, the regulatory protein, GalR, binds two operators, O_E and O_I, located upstream and downstream of the two *gal* promoters. Through a specific protein-protein interaction, GalR "piggybacks" HU, recruiting it to a specific binding site between the two operators (Aki *et al.*, 1996; Kar and Adhya, 2001). HU then bends the DNA between the two operators, forming a loop structure, and a stable, cooperative repressosome, which represses both *gal* promoters (Kar and Adhya, 2001; Lia *et al.*, 2003).

1.5 H-NS, the universal repressor

While other nucleoid-associated proteins, like Fis and IHF, appear to have a number of cellular functions affecting many aspects of DNA metabolism and gene expression both positively and negatively, H-NS appears to act primarily as a transcriptional repressor (Dorman, 2004). Although roles have been identified for H-NS in mediating recombination, it seems to act as a repressor in these cases as well (Kawula and Orndorff, 1991; O'Gara J and Dorman, 2000). It also appears to be involved in indirectly controlling initiation of replication, although little is known about the mechanism involved (Katayama *et al.*, 1996; Atlung and Hansen, 2002). H-NS is a 15.4 kDa protein encoded by a single *hns* gene (Pon *et al.*, 1988), however, similar to HU, many bacteria carry genes encoding related proteins, including the H-NS paralog, StpA

Figure 1.5 A model for the function of HU in the Gal repressosome. Transcription of the *gal* operon is driven by two partially overlapping promoters, *P1* and *P2*, flanked by two operator sequences, O_E and O_I. The regulatory protein, GalR, which binds the operator sequences, is thought to recruit HU to the *hbs* (HU-binding site). Whether GalR interacts with HU before it binds the operators is unknown. HU is then thought to alter local DNA structure so as to promote repressosome formation. Whether HU remains in contact with GalR after recruitment and DNA-binding is unknown.


HU
GalR
P1
O_E
O_I
P2
hbs
GalR recruits HU to hbs
P1
O_E
O_I
P2
hbs
HU binds hbs, promotes repressome formation
O_E
O_I

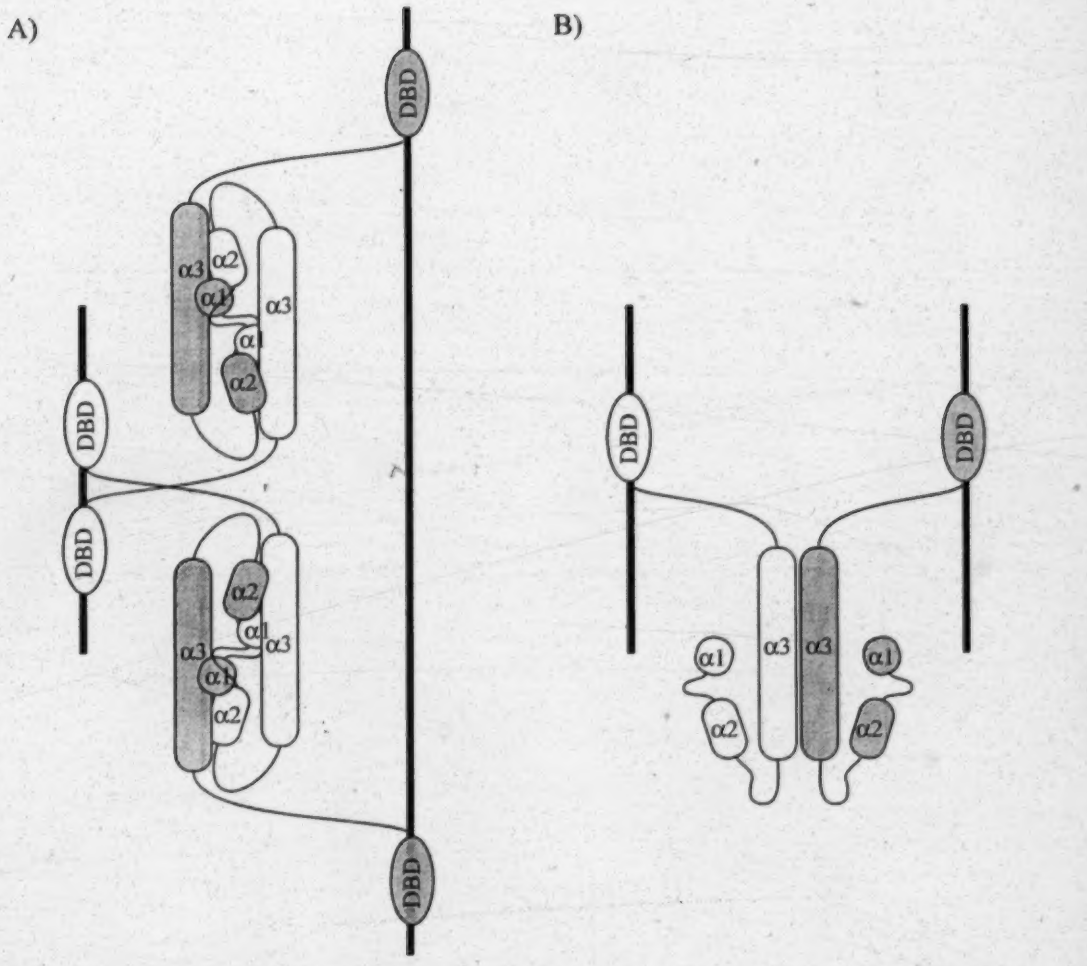
(Zhang and Belfort, 1992), and Hha, an 8.5 kDa protein which is homologous to the oligomerization domain of H-NS (Nieto *et al.*, 1991). Although independent studies have suggested differing expression expression profiles, with H-NS both increasing and decreasing as cell cultures enter stationary phase (Spassky *et al.*, 1984; Ali Azam *et al.*, 1999), thorough analysis suggests that intracellular levels are relatively constant at 20,000 molecules per cell, with *hns* transcription tied to chromosomal replication (Free and Dorman, 1995).

Perhaps the least specific of the four major nucleoid-associated proteins, H-NS binds preferentially to DNA containing regions of intrinsic curvature at sub-saturating concentrations, but binds non-specifically at higher concentrations (Owen-Hughes *et al.*, 1992). H-NS also exhibits RNA-binding activity *in vitro*, with the ability to modulate the stability of at least one small RNA, DsrA (see below), *in vivo* (Brescia *et al.*, 2004), but whether H-NS acts as a chaperone for other transcripts *in vivo* is still unknown. H-NS has the ability to form large oligomeric complexes, a feature which is critical to its ability to recognize intrinsically bent DNA, as well as its ability to bend straight DNA (Spurio *et al.*, 1997). It is thought that oligomerization, along with relatively low specificity, and the ability to induce local DNA bending and perhaps promote additional binding, causes 'nucleation' whereby H-NS spreads across a region of DNA 'zippering' parallel strands of DNA, and 'silencing' transcription from local promoters (Dame *et al.*, 2000; Rimsky *et al.*, 2001; Badaut *et al.*, 2002). This activity is characterized by DNase I footprinting patterns observed in several systems, where H-NS gives specific, discrete footprinting patterns at low concentrations, but eventually nucleates and protects the entire molecule at higher concentrations. This is also observed in atomic force microscopy studies, where

H-NS was observed to 'zipper' plasmid DNA, causing lateral condensation, which is thought to progress to form large globular structures at higher concentrations (Dame *et al.*, 2000).

H-NS is 136 amino acids in length and appears to be comprised of two functional domains. The N-terminal domain consists of residues 1-64, and is thought to be responsible for oligomerization, based on genetic and biochemical studies (Rimsky, 2004). The C-terminal domain consists of residues 90-136, and appears to be responsible for DNA binding. These two domains are thought to be joined by a highly flexible linker (Smyth *et al.*, 2000). The N-terminal oligomerization domain consists of three α-helices: α1 (residues 1-7), α2 (residues 11-18), and α3 (residues 22-46) (Fig. 1.6) (Renzoni *et al.*, 2001). However, models for the actual oligomerization of this domain are quite varied. Early studies suggested that the N-terminal domain formed homotrimers in solution, but formed higher-order complexes at higher concentrations (Renzoni *et al.*, 2001). More recent structural studies have suggested that the domain forms dimers, with the two α3 helices forming a coiled-coil, but how this occurs is still contentious. Analysis of *Salmonella typhimurium* H-NS suggested that the individual monomers are arranged in a parallel fashion, with the α3 helices packed against each other in a coiled-coil, and bound H-NS arranged on the DNA in a head-to-tail fashion (Esposito *et al.*, 2002). However, an independent study of the identical *E. coli* H-NS oligomerization domain determined that the monomers are anti-parallel, with the α2 helix of one monomer packing against the α3 helix of another, and paired α1 helices (Bloch *et al.*, 2003). This structure, resembling two interlocking fish-hooks, is novel for dimeric coiled-coils, and is referred

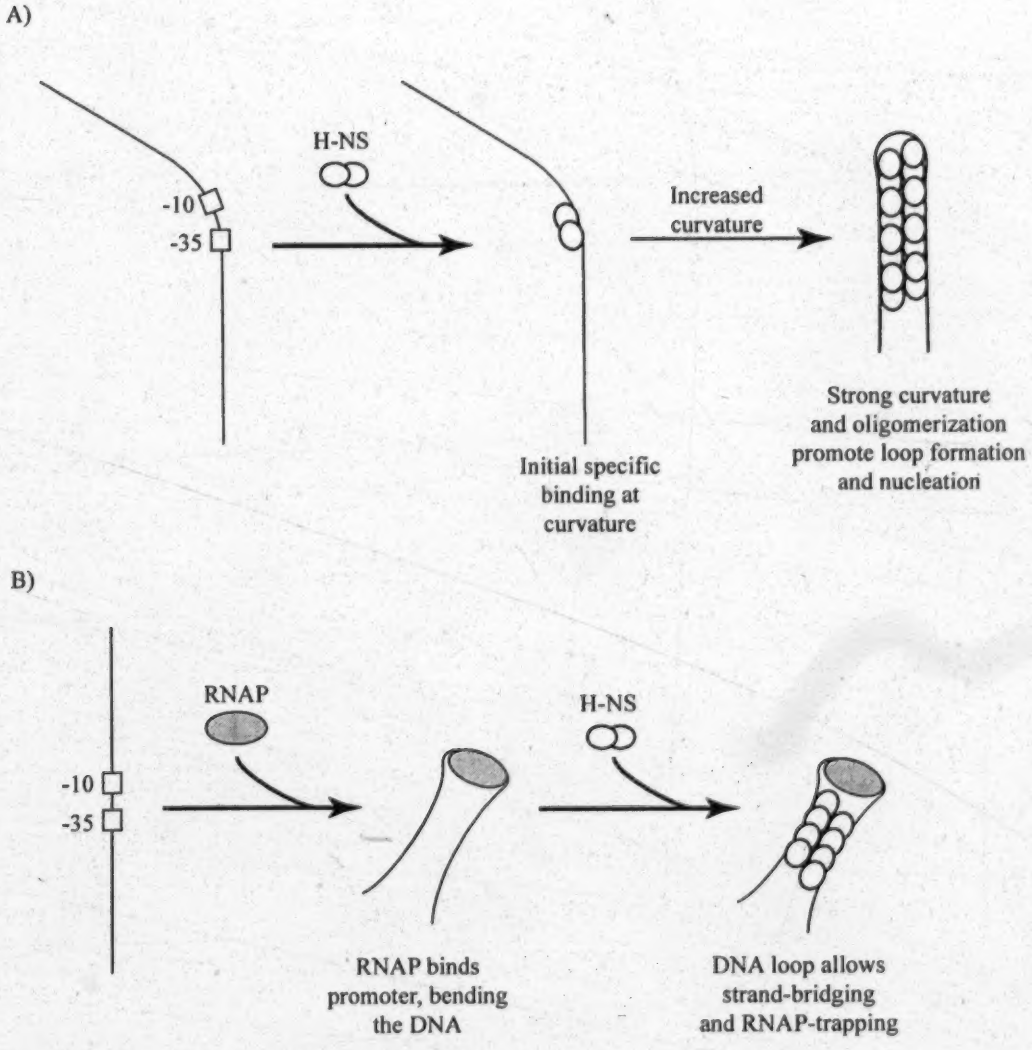
Figure 1.6 H-NS oligomerization and strand bridging. The oligomerization domain consists of three α-helical segments, designated α1 (residues 1-8), α2 residues (residues 12-19), and α3 (residues 23-47), separated by flexible linker regions, with another flexible linker between the oligomerization domain and the DNA-binding domain (DBD; residues 90-137). Two models exist for oligomerization of H-NS. The first suggests that individual oligomerization domains in an H-NS dimer are arranged in an anti-parallel fashion, forming a "hand-shake" fold (A). The alternative model suggests that two H-NS oligomerization domains are arranged in parallel, with the two α3 regions forming a coiled-coil (B). Strand bridging is also explained by two models. The first suggests that individual H-NS dimers, each bound to different regions of DNA, interact and further oligomerize through their flexible linker domains (A). The second suggests that a single dimer binds different regions of the same or different duplexes (B). Adapted by permission from Macmillan Publishers Ltd: *Nat Rev Microbiol.*, 2: H-NS: a universal regulator for a dynamic genome. Dorman, 391-400 (2004).


A)
B)
DBD
DBD
DBD
DBD
DBD
DBD
α1
α2
α3
α1
α2
α3
α1
α2
α3
α1
α2
α3
α1
α2
α3

to as a "hand-shake" fold (Bloch *et al.*, 2003). This structure is supported by the determination of a similar arrangement for the H-NS-like protein in *Vibrio cholerae*, VicH (Cerdan *et al.*, 2003). Oligomerization of whole H-NS is more complex, as genetic studies have shown that both the C-terminal domain and the flexible linker region contribute to oligomerization (Spurio *et al.*, 1997; Stella *et al.*, 2005). These studies suggest that the active form of H-NS *in vivo* is actually tetrameric. Given this, both parallel and anti-parallel models for N-terminal dimerization can be accommodated. If the tetramer is formed by dimerization of two anti-parallel dimers, removal of one monomer from each dimer can produce the parallel arrangement (Stella *et al.*, 2005). How higher-order oligomerization occurs is still unclear, but is a focus of current research, as oligomerization appears to be critical to the activity of H-NS as a repressor.

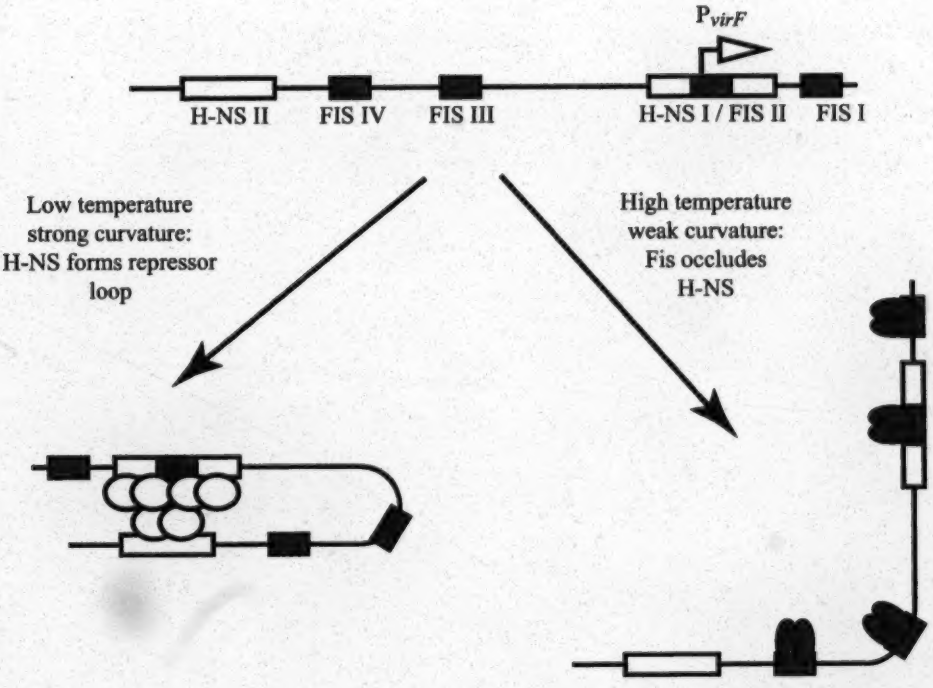
H-NS-mediated repression is thought to occur by one of two mechanisms (Dame *et al.*, 2002). The first mechanism is referred to as the promoter occlusion model, where H-NS binds specifically at a region of intrinsic curvature near a promoter, inducing further local DNA bending and "zippering up" the DNA via its strand bridging activity. This blocks RNA polymerase binding and transcriptional initiation (Fig. 1.7A). The second mechanism is referred to as the RNA polymerase trapping model (Fig. 1.7B). In this case, RNA polymerase binding at a promoter bends local DNA (Coulombe and Burton, 1999) and promotes strand-bridging by H-NS between sites located upstream and downstream of the promoter. This traps the RNA polymerase at the promoter, blocking transcription.

Figure 1.7 Two models for H-NS mediated repression. In the case of the RNA polymerase trapping model, binding of the polymerase bends promoter DNA (A). This promotes strand-bridging by H-NS, closing the DNA loop and trapping the RNA polymerase. In the promoter occlusion model, H-NS binds intrinsically curved DNA at or near the promoter (B). Additional bending may be induced by H-NS or other factors at the curved site, promoting strand-bridging, loop formation, and nucleation. Adapted from *J Biol Chem.* 277: Structural basis for H-NS-mediated trapping of RNA polymerase in the open initiation complex at the *rrnB* P1. Dame *et al.*, 2146-2150 (2002).


A)
H-NS
-10
-35
Increased
curvature
Initial specific
binding at
curvature
Strong curvature
and oligomerization
promote loop formation
and nucleation
B)
RNAP
H-NS
-10
-35
RNAP binds
promoter, bending
the DNA
DNA loop allows
strand-bridging
and RNAP-trapping

One of the best understood models for H-NS-mediated repression is the virulence (*vir*) gene regulation system of *Shigella flexneri*, an organism which can cause dysentery in humans. *S. flexneri* carries a 230-bp plasmid, which encodes a type III secretion system and associated effector proteins required for virulence (Sasakawa *et al.*, 1988). Virulence gene expression is controlled by a regulatory cascade featuring the plasmid-encoded regulatory proteins, VirF and VirB, encoded by separate operons. H-NS represses transcription of both genes at temperatures below 32°C (Maurelli and Sansonetti, 1988). However, at 37°C, *virF* is derepressed. The subsequent increase in intracellular VirF results in activation of *virB*, as VirF is capable of antagonizing H-NS-mediated repression of *virB* in a supercoiling-dependent manner (Tobe *et al.*, 1993). VirB then activates the expression of all downstream virulence genes (Tobe *et al.*, 1991). The critical event in this circuit is the switch between the repressed and derepressed states of *virF*, which appears to involve a number of factors. H-NS binds to two sites at the *virF* promoter, separated by a large bend region (Fig. 1.8). This bend is thought to allow a loop to form between H-NS bound at both sites by H-NS oligomerization. Studies have demonstrated that Fis acts as an activator by binding to two sites overlapping the promoter proximal H-NS site, inhibiting H-NS binding (Falconi *et al.*, 2001). It has also been demonstrated that changes in temperature cause the bend center to shift such that the two H-NS binding sites critical for repression are no longer in phase for efficient oligomerization between the two sites (Prosseda *et al.*, 2004). This is thought to prevent formation of a functional repressor complex and expose Fis binding sites, allowing derepression of *virF* expression (Prosseda *et al.*, 2004). This system highlights many

Figure 1.8 A model for transcriptional regulation of *virF*. The *virF* promoter region features four Fis binding sites, designated FIS I, II, III, and IV, and two H-NS binding sites, designated H-NS I and II. At temperatures below 32°C, a bend centered between H-NS I and H-NS II is relatively strong and stable, allowing strand-bridging by H-NS (indicated by white ovals) and repressor complex formation. However, at 37°C, the bend is destabilized, allowing Fis (indicated by grey ovals) binding and occluding H-NS.


P_{virF}
H-NS II
FIS IV
FIS III
H-NS I / FIS II
FIS I
Low temperature
strong curvature:
H-NS forms repressor
loop
High temperature
weak curvature:
Fis occludes
H-NS

features that have become hallmarks of H-NS-mediated gene repression, including the importance of supercoiling, dynamic curvature, and antagonism by multiple factors.

The fact that these virulence genes are all carried on a plasmid related to the conjugative F factor (Buchrieser *et al.*, 2000) highlights another important point: that H-NS, and, perhaps to a lesser extent, the other nucleoid-associated factors like Fis and IHF, are well suited to regulating mobile genetic elements (Dorman, 2004). This is due to the fact that H-NS binding is not sequence-specific, but structure-specific. Because of this, it can recognize and bind structural elements common to strong promoters, namely intrinsically curved DNA (Rippe *et al.*, 1995). The ability to recognize a wide array of promoters allows a host cell to "sensitize" newly acquired promoters that might otherwise be "blind" to regulatory signals from the host (Dorman, 2004).

This is reinforced by the discovery of several H-NS family proteins carried by mobile genetic elements and the host. These include the H-NS paralogue, StpA (Zhang and Belfort, 1992), and Hha, the oligomerization domain homologue (Nieto *et al.*, 1991), both carried on the host chromosome, as well as the H-NS and Hha homologues carried by mobile elements such as the IncHI plasmids (Beloin *et al.*, 2003).

1.6 Hfq, the global RNA chaperone

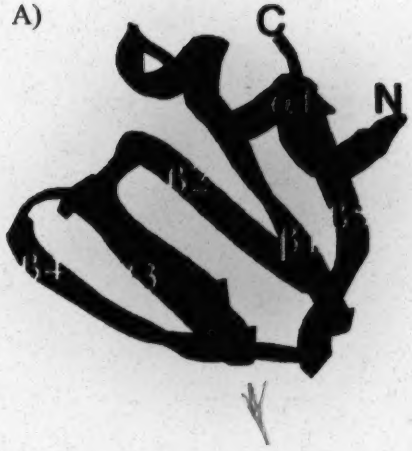
First characterized for its role in the replication of the RNA bacteriophage Qβ (Franze de Fernandez *et al.*, 1968), Hfq (Host factor for phage Qβ replication), or HF-I (Host factor I) as it was originally designated, has been identified as a global regulator of many aspects of RNA metabolism, including RNA phage replication, stability, and translation (Gottesman, 2004; Valentin-Hansen *et al.*, 2004). Although DNA-binding activity has been demonstrated *in vitro*, Hfq appears to act *in vivo* solely as an RNA

binding protein (Azam and Ishihama, 1999; Møller *et al.*, 2002). Hfq binds AU-rich RNA, often flanked by regions of secondary structure, where it introduces structural changes (Møller *et al.*, 2002; Zhang *et al.*, 2002). Although not a nucleoid-associated protein in the purest sense, as it does not bind DNA, Hfq is often included in this group for a number of reasons. First, although it is primarily cytoplasmic, a fraction of cellular Hfq is nucleoid-associated (Azam *et al.*, 2000). Second, Hfq appears to interact directly with H-NS, although the significance of this interaction is not yet understood (Muffler *et al.*, 1996a). Third, its regulatory effect is far-reaching, affecting synthesis of a number of major regulatory proteins, including H-NS (Sledjeski and Gottesman, 1995; Sledjeski *et al.*, 2001). As a result, it has a significant impact on both global gene expression patterns and nucleoid organization.

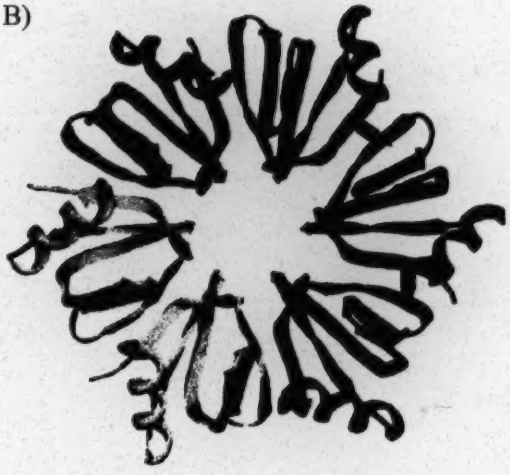
Hfq is a small, 11.2 kDa protein which forms hexameric rings (Fig. 1.9) (Møller *et al.*, 2002; Schumacher *et al.*, 2002; Zhang *et al.*, 2002). It is referred to as a bacterial Sm-like (LSm) protein, due to its homology with the eukaryotic Sm and LSm proteins, which form heteroheptameric complexes that associate with spliceosomal small nuclear RNAs. Hfq possesses two separate surfaces for interacting with RNA, with different target specificities (Mikulecky *et al.*, 2004). The central core of the Hfq ring interacts with AU-rich regions in mRNA and non-coding small RNA, whereas the distal face interacts with poly-A RNA, and is associated with the protein's role in polyadenylation (Hajnsdorf and Regnier, 2000; Mikulecky *et al.*, 2004). Unfortunately, the mechanism of Hfq action is poorly understood. Featuring multiple RNA-binding surfaces with different specificities, Hfq can affect gene expression in several ways. Although its role as a riboregulator, acting in conjunction with sRNAs is well documented (Gottesman, 2004),

Figure 1.9 The structure of *Staphylococcus aureus* Hfq. A ribbon diagram of a single Hfq monomer (A). β1, β2, and β3, make up the Sm1 motif, whereas the Sm2 motif consists of β4 and β5. The Sm1 and Sm2 motifs are characteristic of Sm proteins and strongly conserved. The structure of hexameric Hfq, with each subunit coloured differently (B). Adapted by permission from Macmillan Publishers Ltd: *EMBO J.*, 21: Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. Schumacher *et al.*, 3546-3556 (2002).

A)



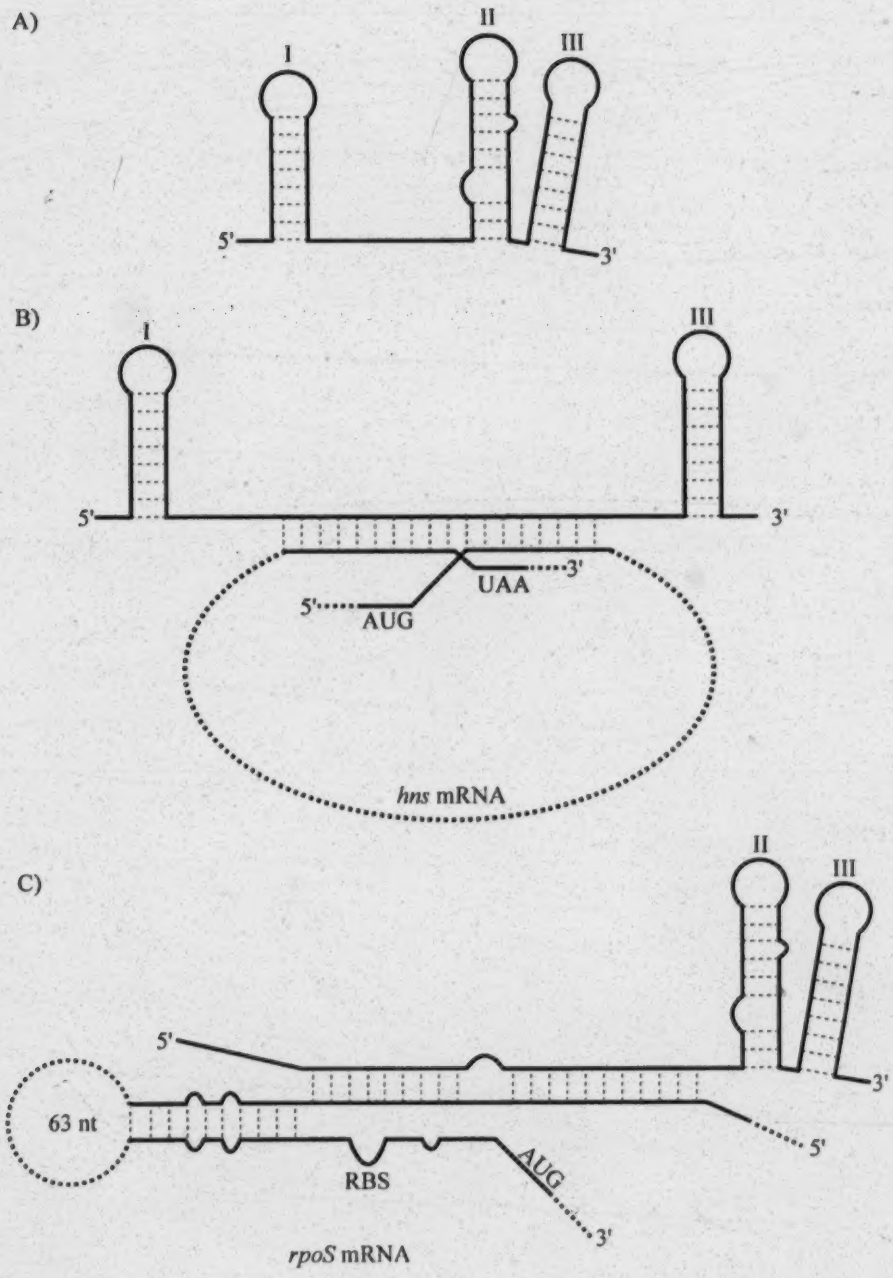
B)



there are other cases where it appears to regulate protein synthesis without assistance from any known sRNAs (Vytvytska *et al.*, 1998; Vecerek *et al.*, 2005). In some cases, it destabilizes or alters RNA secondary structure (Geissmann and Touati, 2004), whereas in others it appears to have no structural effect (Brescia *et al.*, 2003). As Hfq-binding sites and RNase E cleavage sites are similar (Mackie, 1991; Mackie and Genereaux, 1993; McDowall *et al.*, 1994), Hfq has also been associated with modulation of RNase E activity. However, models vary widely, suggesting that Hfq is capable of binding and occluding RNase E (Folichon *et al.*, 2003), as well as binding and altering secondary structure to expose RNase E cleavage sites and promote degradation (Vytvytska *et al.*, 1998), although this is thought to occur indirectly via ribosome occlusion (Vytvytska *et al.*, 2000; Moll *et al.*, 2003b). As a result of these differing effects, there is no clear unifying model for Hfq activity.

The DsrA/*rpoS*/*hns* regulatory system is perhaps the best understood Hfq-regulated circuit (Fig. 1.10). Originally characterized as an H-NS anti-silencer, DsrA is a small, 87 base RNA molecule consisting of three stem-loop structures (Sledjeski and Gottesman, 1995). This anti-silencing property is a result of the ability of DsrA to form a bipartite duplex with both the 5' and 3' tails of the *hns* transcript via stem-loop II, which then promotes degradation of the *hns* mRNA (Lease and Belfort, 2000b). Conversely, DsrA promotes RpoS synthesis by binding to the 5' UTR via stem-loop I (Majdalani *et al.*, 1998). This interaction promotes a structural change in the *rpoS* UTR, exposing the Shine-Dalgarno sequence, which is thought to be otherwise sequestered by a *cis*-acting translational operator, inhibiting translation (Brown and Elliott, 1997). The interaction between DsrA and *rpoS* also stabilizes the *rpoS* transcript (Lease and Belfort, 2000a).

Figure 1.10 Mechanisms of DsrA-mediated regulation. DsrA consists of three stem-loops (A). Stem-loops I and II are involved in RNA-RNA interactions, whereas stem-loop III is involved in stability. Stem-loop II forms a limited heteroduplex with the 3'and 5' termini of the *hns* mRNA, promoting degradation of the transcript (B). Stem-loop I forms a limited heteroduplex with the 5' UTR of the *rpoS* mRNA, exposing the ribosome binding site that is otherwise occluded by the secondary structure of the RNA (C). Adapted with permission from Blackwell Publishing: *Mol Microbiol.* 38: Riboregulation by DsrA RNA: trans-actions for global economy. Lease and Belfort, 667-672 (2000).


A)
I
II
III
5'
3'
B)
I
III
5'
3'
UAA
3'
5'
AUG
hns mRNA
C)
II
III
5'
3'
63 nt
5'
RBS
AUG
3'
rpoS mRNA

Hfq binds DsrA and promotes both stability and activity (Sledjeski *et al.*, 2001), but it does not cause any changes in secondary structure (Brescia *et al.*, 2003). This indicates that Hfq, DsrA, and their mRNA targets form a higher order-complex, as Hfq activity cannot be explained by simple structural alterations of the sRNA. DsrA-Hfq-mediated regulation demonstrates some common themes of sRNA-Hfq-mediated regulation. DsrA, like many small RNAs, is capable of interacting with multiple mRNA targets, with multiple independent interaction surfaces. Furthermore, sRNAs do not have to be perfectly complementary antisense RNAs (Gottesman, 2004). Rather, many characterized sRNAs are not genetically linked from their target genes, and display only partial homology (Gottesman, 2004). In the case of *rpoS*, the target UTR is only complementary to 20 non-contiguous bases of DsrA (Lease *et al.*, 1998). This degeneracy allows interactions with multiple mRNA targets, and may be important in allowing alterations in secondary structure during heteroduplex formation between the sRNA and the target mRNA.

Another relatively well-understood Hfq-regulated system is OmpA synthesis. Unlike Hfq-mediated regulation of *hns* and *rpoS*, there is no known sRNA involved. In this case, Hfq binds to the 5' UTR of the *ompA* transcript and promotes its degradation at slow growth rates by RNase E (Vytvytska *et al.*, 1998). Hfq acts as an RNA chaperone, inducing a stable structural alteration in the 5' UTR (Moll *et al.*, 2003b). This structural alteration then blocks 30S ribosome binding, preventing translation and stabilization of the transcript (Moll *et al.*, 2003a; Moll *et al.*, 2003b). Similar findings have also been reported for the *hfq* mRNA 5' UTR, where Hfq acts as an RNA chaperone, inducing a

structural change which inhibits 30S ribosome binding and translation (Vecerek *et al.*, 2005).

1.7 Nucleoid-associated proteins and horizontal gene transfer

Horizontal transfer allows the exchange of genetic material between bacterial cells by three possible mechanisms. The first is transformation, whereby naked DNA is taken up from the environment. The second mechanism is transduction, whereby DNA is packaged and transferred between bacteria by a bacteriophage. The final mechanism is conjugation, which is, in effect, a fusion between a Type IV secretion system and a specialized DNA replication system, allowing DNA to be transferred between bacterial cells in close physical contact (Llosa *et al.*, 2002). Conjugation often involves the transfer of plasmids, genetic elements which can exist independently of the chromosome, encoding their own replication and partitioning systems in addition to the cellular machinery necessary for transfer. Although this mechanism of conjugation is the focus of this thesis, other systems, involving conjugative plasmid-chromosome co-integrates and conjugative transposons also exist (Clewell *et al.*, 1995). In addition to genes responsible for replication, partitioning, and transfer, plasmids can carry genes encoding a number of potentially advantageous traits, including, but not limited to, antibiotic resistance, alternative metabolic pathways, and virulence systems.

While the ability to transfer large segments of genetic material between bacterial cells via these systems may be advantageous in allowing more rapid adaptation to selective pressure than would occur by the simple accumulation of point mutations (Davison, 1999), it also presents additional problems. Newly acquired genes, especially those acquired via interspecies transfer, may be 'blind' to host regulatory signals,

particularly those involving highly-specific regulatory factors. This presents the question of whether general gene regulation systems exist to control the expression of non-essential genes, particularly during periods of physiological and nutritional stress.

While nucleoid-associated factors have long been known to be involved in transfer events, the full significance of this relationship was not always clear. Although the initial work on both IHF and Fis focused on their architectural roles in phage integration and excision (Robertson and Nash, 1988; Ball and Johnson, 1991a), more recent studies have also identified regulatory roles for these proteins. Fis and H-NS appear to be involved in the regulation of a number of virulence gene systems which are thought to have been acquired horizontally, such as the plasmid-encoded *vir* systems in *S. flexneri* and enteroinvasive *E. coli* (Falconi *et al.*, 2001), and the SPI-1 pathogenicity island in *S. typhimurium* (Wilson *et al.*, 2001; Schechter *et al.*, 2003).

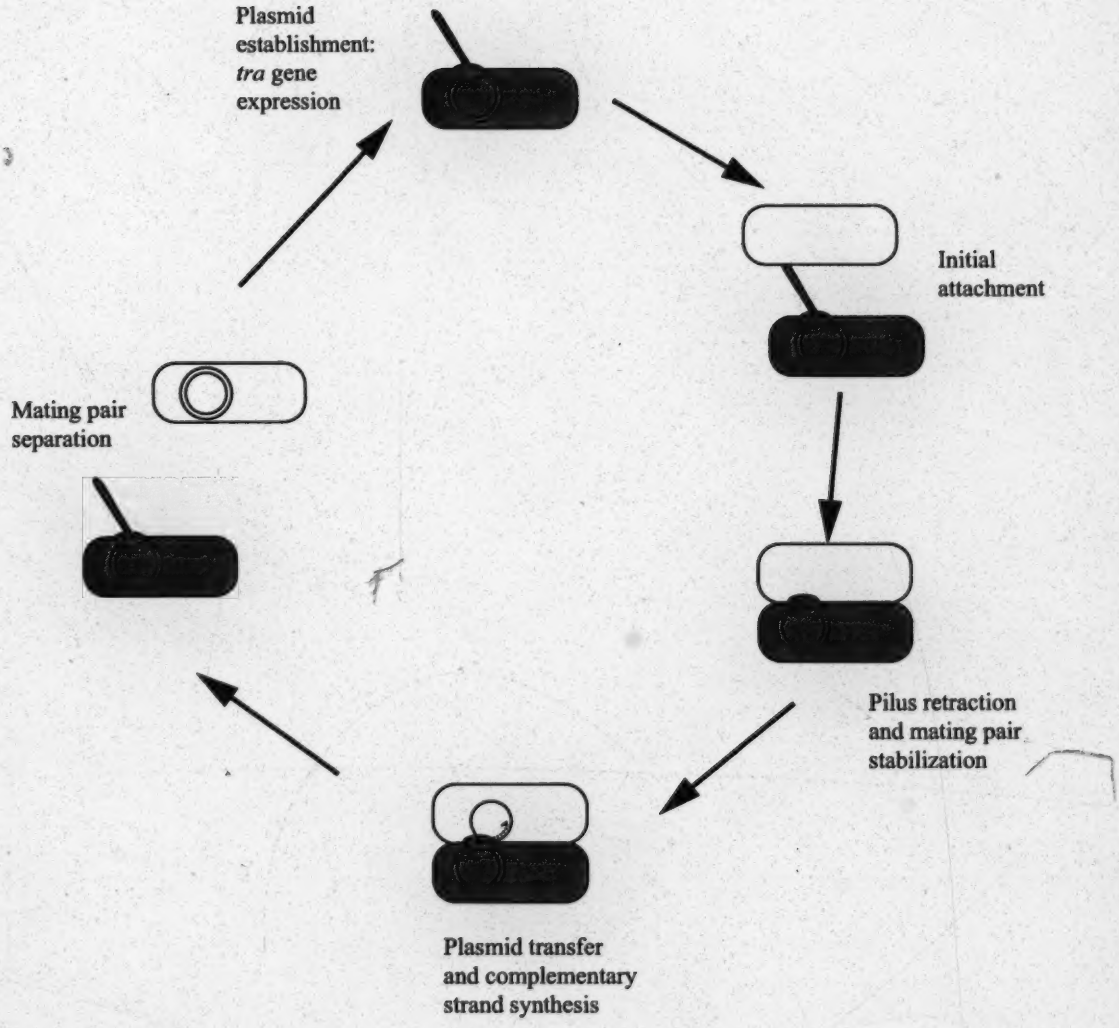
As described above, these proteins are uniquely suited to regulating a wide array of genes, including those acquired by horizontal transfer, due to their promiscuous target-binding activity. By recognizing general structural elements, rather than specific sequences, they are capable of binding many promoters, particularly when that structural element is common to active promoters, as is the case with H-NS. This allows the host cell to control otherwise "blind" expression from these newly acquired genes. The significance of this is further reinforced by the discovery of proteins encoded by these mobile genetic elements which are capable of interacting directly with these host factors. The first clear example of this is gp5.5, encoded the T7 bacteriophage, which binds H-NS directly, inhibiting H-NS-mediated repression of both T7 and *E. coli* RNA polymerases (Liu and Richardson, 1993). Many mobile genetic elements also encode homologs to

these host factors. Ler, encoded by the LEE (locus for enterocyte effacement) pathogenicity island in enteropathogenic *E. coli*, has extensive homology with H-NS DNA binding domain, and antagonizes H-NS-mediated silencing of the *LEE* operons (Elliott *et al.*, 1998; Bustamante *et al.*, 2001). A number of conjugative plasmids encode H-NS and Hha homologues, some of which have been shown to be involved in the regulation of plasmid transfer (Forns *et al.*, 2005).

1.8 A brief overview of the F plasmid

Since the first studies of conjugation (Lederberg and Tatum, 1946), the 100 kb F plasmid has become a model system for horizontal gene transfer in general, and bacterial conjugative plasmids in particular. Conjugation is made possible by a large, multi-component, protein complex belonging to the type IV secretion system family, referred to as the transferosome, which spans the bacterial cell membrane, forming a channel to facilitate DNA transfer into a suitable recipient cell (Christie, 2001; Lawley *et al.*, 2003). The transfer event consists of several steps, but is thought to be initiated by contact between the conjugative pilus and a recipient cell (Fig. 1.11). This contact induces pilus retraction and stable mating pair formation, where the donor and recipient cell membranes come into close contact, and remain so for the duration of the transfer event (Novotny and Fives-Taylor, 1974; Durrenberger *et al.*, 1991). Once a stable mating pair is formed, transfer of the plasmid is thought to be initiated by a signal between the transferosome and a nucleosomal complex called the relaxosome, which is responsible for processing the plasmid DNA for transfer (see below), via a coupling protein (Llosa *et al.*, 2002). This coupling protein, designated TraD in the F plasmid, is located on the inner face of the pore, and is thought to serve as a pump to drive DNA transfer through

Figure 1.11 The bacterial conjugation cycle. Donor cells that are expressing transfer genes and are competent for transfer (gray cells) make initial contact with suitable recipient cells (white cells) via a conjugative pilus. The pilus then retracts, bringing the cells into close physical contact, forming a stable mating pair. DNA containing an origin of transfer is then nicked and unwound as one strand is spooled through a conjugative pore into the recipient cell. Complementary strands of DNA are synthesized simultaneously in both the donor and the recipient cell. Following completion of transfer, the mating pair dissociates. The new transconjugant cell undergoes a period of establishment and transfer gene expression, becoming a mature donor.


Plasmid establishment: *tra* gene expression
Initial attachment
Pilus retraction and mating pair stabilization
Plasmid transfer and complementary strand synthesis
Mating pair separation

the conjugative pore (Llosa *et al.*, 2002). Following completion of plasmid transfer, the mating pair dissociates, which is thought to be the result of a group of plasmid-encoded proteins referred to as surface exclusion proteins, which prevent mating between donor cells (Achtman *et al.*, 1977).

1.9 The F plasmid relaxosome

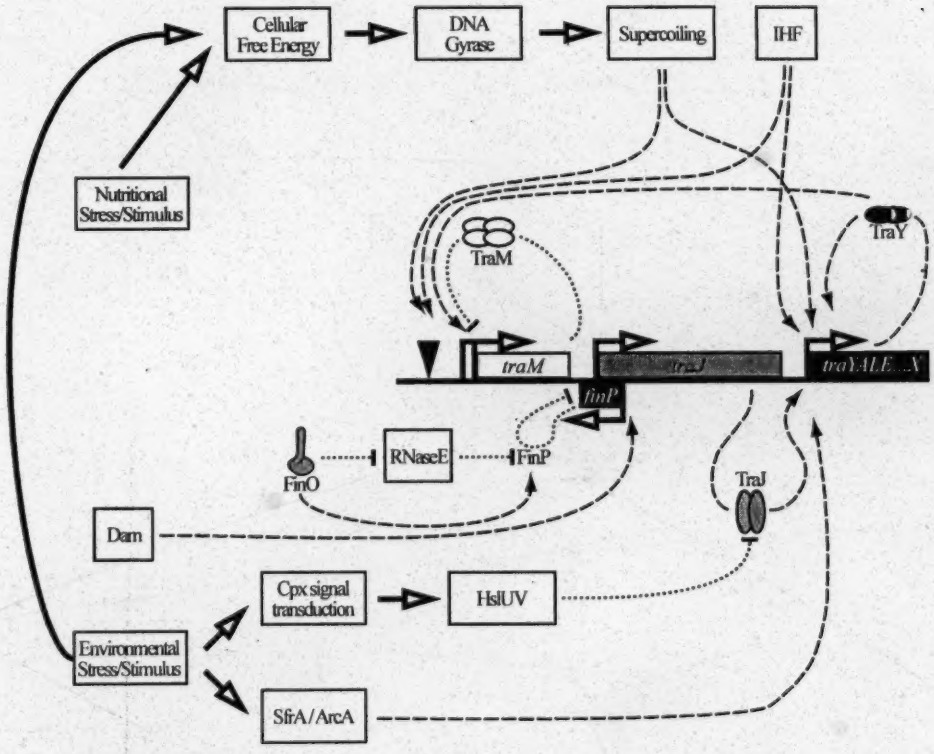
Transfer requires that the plasmid DNA be nicked and unwound, allowing a single strand of plasmid DNA to be generated in a 5' to 3' direction via rolling-circle replication, which is then fed through the conjugative pore (Zechner *et al.*, 2000). This processing event is mediated by a nucleosomal complex referred to as the relaxosome. This complex forms at a region on the plasmid called the origin of transfer (*oriT*) and features both plasmid and host-encoded factors. IHF and TraY, a plasmid-encoded factor, bind *oriT* (Lahue and Matson, 1990; Tsai *et al.*, 1990; Nelson *et al.*, 1993; Gao *et al.*, 1994), where they are thought to recruit the plasmid-encoded nickase/helicase, TraI, by re-structuring local DNA architecture to allow TraI binding and activity (Howard *et al.*, 1995; Nelson *et al.*, 1995). TraI then generates a site-specific nick in the DNA at *nic* via a transesterification reaction whereby the newly generated 5' end is covalently linked to a tyrosine residue in TraI (Matson and Morton, 1991; Reygers *et al.*, 1991; Matson *et al.*, 1993; Byrd and Matson, 1997). TraI then unwinds the DNA for transfer while a replacement strand is synthesized (Lahue and Matson, 1988). TraI is also capable of reversing the transesterification reaction using the nucleophile provided by the free 3'-OH terminus, which is thought to be important in signaling the end of transfer (Sherman and Matson, 1994). A third plasmid-encoded protein, TraM, is also involved in the relaxosome, although it is not essential for TraI activity. TraM also binds *oriT*, but is

thought to be involved in transmitting the signal for transfer between the relaxosome and the transferosome via the coupling protein, TraD (Disque-Kochem and Dreiseikelmann, 1997; Fekete and Frost, 2002; Lu and Frost, 2005).

1.10 F plasmid *tra* gene regulation

All of the genes necessary for F plasmid transfer lie within a single region on the plasmid, with *oriT* located at one end. Immediately downstream of *oriT* is the monocistronic *traM* operon, followed by another monocistronic operon, *traJ*, which encodes an essential activator for plasmid transfer gene expression (Willetts, 1977). The remaining transfer genes are all located within a single 33 kb polycistronic transfer (*tra*) operon (Frost *et al.*, 1994). F plasmid transfer gene expression, and hence transfer, is extremely growth-phase dependent, limited to exponential phase growth (Frost and Manchak, 1998). Transfer gene expression requires the primary activator, TraJ, which activates transcription from the *tra* operon promoter, P_Y (Fig 1.12) (Willetts, 1977; Silverman *et al.*, 1991a). However, the mechanism of TraJ-mediated activation is unclear. F-like TraJ proteins exhibit little homology to each other, and do not exhibit significant homology to any other known proteins (Frost *et al.*, 1994). Furthermore, despite possessing a predicted helix-bend-helix DNA-binding motif, DNA binding activity has only been demonstrated once, under acidic conditions, with TraJ from the F-like plasmid, R100 (Taki *et al.*, 1998). DNA-binding activity for F TraJ has not been demonstrated. However, indirect genetic and biochemical studies have suggested a model for TraJ activity. Genetic analysis has demonstrated that deletion of sequences upstream of the -78 position relative to P_Y restored promoter activity in the absence of TraJ, suggesting that regulation of P_Y was dependent on sequence context (Silverman *et*

Figure 1.12 The basic *tra* regulatory circuit. The F plasmid regulatory circuit consists of three plasmid-encoded regulatory factors: TraJ, TraM, and TraY. The primary activator, TraJ, upregulates the polycistronic *tra* operon, which encodes most of the proteins necessary for transfer, including TraY. TraY further regulates *tra* operon expression and activates transcription of *traM*. TraM is then thought to repress its own expression. The FinOP antisense RNA system represses TraJ synthesis in F-like plasmids, but is inactive in F due to the insertion of an IS*3* element in *finO*. Experimental data suggests regulatory roles for IHF, ArcA, and the CpxA/R two-component signal transduction system as well, although the mechanisms are unclear. Negative effects are indicated by short dashed lines ending in a bar. Positive effects are indicated by long dashed lines ending in an arrowhead. General host regulatory pathways are indicated by solid, bold lines.


Cellular Free Energy
DNA Gyrase
Supercoiling
IHF
Nutritional Stress/Stimulus
TraM
TraY
traM
traJ
traYALE...X
finP
RNaseE
FinP
FinO
TraJ
Dam
Cpx signal transduction
HslUV
Environmental Stress/Stimulus
SfrA/ArcA

al., 1991b). Subsequent studies demonstrated that transcription from P_Y *in vitro* required a supercoiled template (Gaudin and Silverman, 1993). However, alterations in the promoter sequence which eliminated a requirement for supercoiling, also eliminated a requirement for TraJ (Gaudin and Silverman, 1993). From this, it was hypothesized that TraJ activated transcription from P_Y by opposing the formation of a repressive nucleosomal complex upstream of P_Y which altered local supercoiling. The first gene in the *tra* operon encodes the relaxosome component, TraY (Frost *et al.*, 1994). TraY also appears to have a role regulating *tra* gene expression, although its exact function is unknown. A TraY binding site, *sbyB*, has been identified immediately downstream of P_Y (Nelson *et al.*, 1993), but conflicting reports have indicated both positive (Maneewannakul *et al.*, 1996; Silverman and Sholl, 1996), and negative (Taki *et al.*, 1998) TraY-dependent effects on transcription from P_Y. TraY also activates transcription of *traM* from its promoters P_{M1} and P_{M2}, collectively referred to as P_M, via two binding sites at *oriT*: *sbyA*, which is required for nicking, and *sbyC* (Lahue and Matson, 1990; Fu *et al.*, 1991; Nelson *et al.*, 1993; Penfold *et al.*, 1996). TraM completes the plasmid-encoded regulatory circuit by acting as an autorepressor, binding to three sites, *sbmA*, *B*, and *C*, located between *oriT* and P_M (Penfold *et al.*, 1996).

Although TraJ, TraY, and TraM comprise the core of the plasmid regulatory circuit, additional regulatory input is provided by a number of plasmid and host-encoded factors. F-like plasmids, with the exception of F, are regulated by the FinOP antisense RNA system (Finnegan and Willetts, 1972). FinP is a small, 79 base antisense RNA complementary to the 5' UTR of the *traJ* transcript, and is thought to act by forming a heteroduplex with the *traJ* transcript, occluding the ribosome binding site and inhibiting

translation (van Biesen *et al.*, 1993; Koraimann *et al.*, 1996). FinO is an RNA chaperone which is essential for FinP activity, preventing degradation by RNase E and promoting duplex formation (Jerome *et al.*, 1999; Arthur *et al.*, 2003). However, in the case of the F plasmid, *finO*, located at the *oriT*-distal end of the transfer region, downstream of the *tra* operon, is disrupted by an IS*3* element, resulting in derepression of TraJ synthesis, and hence, plasmid transfer (Cheah and Skurray, 1986).

TraJ also appears to be subject to post-translational regulation, as studies have demonstrated that activation of the Cpx two-component signal transduction system, which is associated with cell envelope stress (Raivio and Silhavy, 2001), results in a decrease in TraJ stability (Silverman *et al.*, 1993; Gubbins *et al.*, 2002). Although the mechanism is still unclear, Cpx-mediated destabilization of TraJ is thought to be due to an increased synthesis of host proteases, possibly HslUV, which then degrade TraJ (Lau and Frost, unpublished results).

F *tra* gene transcription is also subject to host-mediated regulation. The response regulator of the Arc two-component signal transduction system, ArcA, is known to bind DNA upstream of P_Y in the F-like plasmid, R100 (Strohmaier *et al.*, 1998), and is thought to activate *tra* gene expression in a TraJ-dependent manner (Silverman *et al.*, 1991a), although the mechanism is unclear. Earlier studies have also hinted at a possible role for IHF in regulating *tra* gene expression, but independent studies have provided contradictory results, arguing for both positive and negative regulatory roles (Gamas *et al.*, 1987; Dempsey and Fee, 1990; Silverman *et al.*, 1991a; Abo and Ohtsubo, 1993).

1.11 Summary and research objectives

Despite having characterized the core plasmid regulatory circuit, featuring TraJ, TraY, and TraM, major questions about *tra* gene regulation remain unanswered. While many previous studies have examined *tra* gene regulation under optimum growth conditions during exponential growth, these conditions are clearly not representative of the bacterial life cycle as a whole, and in particular, of bacterial cells in their native environment. Why does *tra* gene expression shut off in stationary phase? Or, conversely, why does *tra* gene expression turn on briefly in exponential phase? How does TraJ regulate P_Y? What regulates *traJ* expression?

The primary objective of this thesis was to identify the signal responsible for repressing F plasmid transfer and transfer gene expression during entry into stationary phase. The results presented in Chapters 2 and 3 indicate that host-encoded nucleoid-associated protein, H-NS, plays a central role in repressing *tra* gene expression. H-NS was shown to bind regions of predicted intrinsic curvature at P_J, P_Y, and P_M. Furthermore, *tra* gene expression is derepressed in *hns* donor cells entering stationary phase. Genetic analysis indicated that this repression is highly context dependent, requiring a large segment of F plasmid DNA to occur, suggesting that repression was due to a regional silencing complex.

The second objective of this thesis was to determine whether plasmid-encoded factors activate *tra* gene expression by opposing this silencing event. Results presented in Chapter 3 demonstrate that neither TraJ nor TraY are necessary for *tra* gene expression in an *hns* host. This suggests that these proteins function to disrupt H-NS silencing.

The third objective of this thesis was to identify any other nucleoid-associated proteins involved in *tra* gene regulation. Results presented in chapter 4 suggest that Hfq

also acts as a repressor of *tra* gene expression by promoting degradation of transcripts containing the *traJ* 5' UTR. However, Hfq does not appear to be involved in FinOP-mediated repression. Chapter 5 presents the results of a comprehensive screen of the remaining nucleoid-associated proteins, including IHF, Fis, HUα, HUβ, and the H-NS paralogue, StpA. In this study, only IHF had a clear effect on *tra* gene expression, up-regulating each of the Tra regulatory proteins. These results are likely representative of host-mediated control of mobile genetic elements in general, as promiscuous host factors, present at high intracellular concentrations, are capable of regulating a variety of targets.

Chapter 2: Materials and Methods

2.1 Bacterial strains and growth conditions

Bacterial strains are described in Table 2.1. All cultures were grown in Luria-Bertani (LB; 1% (w/v) Difco Tryptone, 0.5% (w/v) Difco Yeast Extract, 1% (w/v) NaCl) broth or agar plates at 37° C unless indicated otherwise. MOPS media was prepared as previously described (Neidhardt *et al.*, 1974), with the appropriate carbon source, glucose, glycerol, or acetate, added to a final concentration of 0.2 % (w/v). L1 synthetic solid medium contains 1 x M9 (minimal salts: 48 mM $Na_2HPO_4 \cdot 7H_2O$, 22 mM KH_2PO_4, 8 mM NaCl, and 19 mM NH_4Cl), 0.4% (w/v) lactose, 5 mM $MgSO_4$, and 1.5% (w/v) agar. Antibiotics were used at the following concentrations on selective media: ampicillin, 25 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 200 μg/ml; and tetracycline, 10 μg/ml. In all experiments using the *hns* strain, PD32, the mutant strain was freshly made via P1 phage transduction, or grown from glycerol stocks stored at -80° C that were freshly transduced before storage. Strains RW1, RW2, RW3, RW4, RW7, and RW10 were constructed by P1 phage transduction as indicated in Table 2.1

2.2 Recombinant DNA techniques and plasmid construction

DNA manipulation and amplification via polymerase chain reaction (PCR) were performed using standard protocols as previously described (Sambrook *et al.*, 1989). All restriction enzymes, T4 DNA ligase, alkaline phosphatase, polynucleotide kinase, and Klenow fragment were supplied by Roche Diagnostics. PCR was performed using Vent polymerase (New England Biolabs). DNA sequencing was performed using the DYEnamic ET terminator cycle sequencing kit (GE Healthcare) on an Applied Biosystems 373-S DNA sequencer unless otherwise indicated. DNA sequences were

Table 2.1 Bacterial strains used in this study.

Strain	Genotype	Source or reference
AJW1939	AJW678 *ackA*::Km	A. Wolfe, Loyola University (Kumari *et al.*, 2000)
AJW2013	AJW678 Δ(*ackA pta hisJ hisP dhu*) *zej223*-Tn*10*	A. Wolfe, Loyola University (Wolfe *et al.*, 2003)
AM111	MC4100 *hfq1*::Ω, Km^R	R. Hengge, Freie Universitat Berlin (Tsui *et al.*, 1994; Muffler *et al.*, 1996b)
AM112	MC4100 *hfq2*::Ω, Km^R	R. Hengge, Freie Universitat Berlin (Tsui *et al.*, 1994; Muffler *et al.*, 1996b)
BSN5	MC4100 *stpA*::Km	B. Uhlin, Umea University (Sonden and Uhlin, 1996)
ED24	F^- Lac^- Spc^r	Achtman *et al.* (1971)
JC3272	F^- *lacΔX74 gal his trp lys rpsL tsx*	Achtman *et al.* (1971)
JRY764	C600 *hupB*::Km	J. Rouviere-Yaniv, Institut de Biologie Physico-Chemique (Huisman *et al.*, 1989)
JRY765	C600 *hupA*::Cm	J. Rouviere-Yaniv, Institut de Biologie Physico-Chemique (Huisman *et al.*, 1989)
MC251	*ara* Δ(*lac-pro*) *gyrA metB argE rift hi supF* Δ82(*himA*)::Tn*10*	Gamas *et al.* (1986)
MC4100	F^- *araD139 Δ(argF-lac)U169 rpsL150*(Str^r) *relA1flb5301 deoC1 ptsF25 rbsR*	Casadaban (1976)
PD32	MC4100 *hns-206*::Ap^r	Dersch *et al.* (1993)

Table 2.1 continued

Strain	Genotype	Source or reference
RLG1863	RLG851 *fis*::*kan-767*	R. Gourse, University of Wisconsin (Appleman *et al.*, 1998)
RW1	MC4100 *fis*::*kan-767*	This work: (P1)[a] RLG1863→MC4100 (Km)
RW2	MC4100 Δ82(*himA*)::Tn*10*	This work: (P1) MC251→MC4100 (Tc)
RW3	MC4100 *hupB*::Km	This work: (P1) JRY764→MC4100 (Km)
RW4	MC4100 *hupA*::Cm	This work: (P1) JRY765→MC4100 (Cm)
RW7	JC3272 *ackA*::Km	This work: (P1) AJW1939→JC3272 (Km)
RW10	JC3272 Δ(*ackA pta hisJ hisP dhu*) *zej223*-Tn*10*	This work: (P1) AJW2013→JC3272 (Tc/Ace$^-$)

[a] (P1) indicates a strain generated by P1 transduction. The lysate source is indicated first, followed by the infected strain and the selected marker.

analyzed on Genetools software (Biotools). Oligonucleotides were synthesized in the Department of Biological Sciences on an Applied Biosystems 392 DNA synthesizer, or purchased from GenoSys (Sigma-Aldrich). Plasmid DNA was purified using the Birnboim-Doly method (Birnboim and Doly, 1979), or with Qiagen Miniprep and Maxiprep kits. Plasmid transformations were performed using $CaCl_2$ competent cells (Sambrook et al., 1989). All small DNA fragments (less than approximately 400 bp) were gel purified on an 8% TBE (90 mM Tris-HCl, 90 mM H_3BO_3, 2 mM EDTA, pH 8.0) polyacrylamide gel. Fragments were cut out of the gel, eluted overnight at 37°C in 500 mM ammonium acetate and 1 mM EDTA. Following the elution step, the fragments were extracted once with an equal volume of 1:1 phenol:chloroform, and once with an equal volume of chloroform. Purified fragments were then precipitated by adding 1 µl glycogen (Roche), 0.1 volume 3M sodium acetate, and 2 volumes of 95% ethanol. DNA pellets were then washed twice with 70% ethanol and air-dryed. All oligonucleotides larger than 30 bases were purified as above, except the TBE-acrylamide gel contained 8 M urea. Larger DNA fragments were purified from agarose gels using the Qiagen Gel Extraction Kit. All plasmids used in this study are described in Table 2.2. All oligonucleotides used in this study are described in Table 2.3.

Transcriptional fusion constructs were constructed by cloning promoter fragments into pJLac101, a low-copy number, RK2/RP4 replicon-based *lacZ* transcriptional fusion vector. pJLac101 is a variant of the promoter probe vector, pPR9TT (Santos *et al.*, 2001), which has had the translational start of *traM* fused to *lacZ*, to allow its use as a transcriptional fusion vector. pRWPY101 was generated by cloning a fragment amplified

Table 2.2 Plasmids used in this study.

Plasmid	Description	Reference
F*lac*	*tra*$^+$ *finO*$^-$, transfer-derepressed F derivative, *lac*$^+$	Achtman *et al.* (1971)
F*lac traJ90*	*traJ*, *lac*$^+$ F derivative	Achtman *et al.* (1971)
pBR322	General cloning vector	New England Biolabs
pJLac101	pPR9TT-1-derived transcriptional fusion-based promoter assessment plasmid	Will *et al.* (2004)
pJLac104	pJLac101 with F P_M upstream of the reporter gene	Will *et al.* (2004)
pJLac106	pJLac101 with F P_J (including *finP* and P_{finP} in the opposite direction) upstream of the reporter gene	Will *et al.* (2004)
pJLac107	pJLac101 with F P_J (including *finP* but no P_{finP} in the opposite direction) upstream of the reporter gene	Will *et al.* (2004)
pJLac110	pJLac101 with F P_M, *traM*, T_{traM}, P_J (including *finP* and P_{finP} in the opposite direction) upstream of the reporter gene	Will *et al.* (2004)
pJLac122	pJLac101 with F P_M, *traM*, T_{traM}, P_J (including *finP* but no P_{finP} in the opposite direction) upstream of the reporter gene	Will *et al.* (2004)
pJLOY401	pBluescript KS+ with an F fragment from *oriT* to P_Y	Will *et al.* (2004)
pLJ5-13	pUC19 with a *T7Φ10-finP* fusion	Jerome *et al.* (1999)
pNY300	pUC18 with F *oriT* and *traM*	Frost *et al.* (1989)
pOX38-Km	*tra*$^+$ *finO*$^-$, transfer-derepressed F derivative, Km^R	Chandler and Galas (1983)
pOX38-Tc	*tra*$^+$ *finO*$^-$, transfer-derepressed F derivative, Tc^R	Anthony *et al.* (1994)
pOX38-*traY244*	*traY*::Km^R *finO*$^-$ F derivative	Maneewannakul *et al.* (1996)
pRWPY101	pJLac101 with F P_Y (including the *traY* ORF and part of *traJ*) fused to *lacZ*	This work

Table 2.2 continued

Plasmid	Description	Reference
pRWPY102	pJLac101 with F P_Y (including the *traY* ORF) fused to *lacZ*	This work
pRWPY103	pJLac101 with F P_Y (including part of *traJ*) fused to *lacZ*	This work
pSnO104	pACYC184 containing *finO* from R6-5	Lee *et al.* (1992)
pTE607	pET3a encoding a C-terminal six-His-tagged Hfq	T. Elliott (West Virginia University)

Table 2.3 Oligonucleotides used in this study

Oligonucleotide	Sequence
23SR3	5'AAGGTTAAGCCTCACGGTTC3'
A2426	5'AACACGCATCTCTGATATGCGAC3'
A2428	5'CGCTTCTGTTACTTGCCTC3'
LFR21	5'GAGGTTCCTATGTAT3'
RWI58	5'GCGCAGATCTCGCGTTAATAAGGTGTTAATAAAAT3'
RWI59	5'GCGCAGATCTGCTGCTCATGTTCGTCATAAAGA3'
RWI62	5'GCGCGGTACCCTGCCCTGTTAAACTTCGGATAGC3'
RWI63	5'GCGCGGTACCTTTTTGACGGGCGCAGAAGCACC3'
RWI65	5'CCTCATGTCTCCGGAAATTCAAAGTT3'
RWI68	5'TTTCCAGCAGGATCTATTTGACGAGCA3'
RWI78	5'AGCGACTTACCATAGATATTCCCTGCCCTGTTAAACTT CG3'
RWI79	5'GCACCGGAATCAGTCATAATGCT3'
SPE5-ext	5'CGTTCCATCTCAGATGATACCTTCTCCCTGATATCTTCA ACCATATTGGC3'
SPE8	5'CATAGGCATCATTGCTGATATACAG3'
TvB14	5'CCTGAATAACTGCCGTCAG3'
TvB15	5'TCGAATTCTAATACGACTCACTATAGACGTGGTTAATG CCACG3'

by polymerase chain reaction (PCR) from pOX38-Tc using the primers RWI59 and RWI63 into the BglII and KpnI sites of pJLac101. pRWPY102 and pRWPY103 were similarly constructed using the primer pairs RWI58 and RWI63, and RWI59 and RWI62, respectively.

2.3 Mating assays

Standing overnight cultures of donor strains were diluted 1:200 in fresh LB broth and incubated with shaking at 37°C. For the growth phase mating assays, at the indicated times, samples equivalent to 0.1 OD_{600} were removed and mated for 1 hour at 37° C with a similar amount of the recipient, ED24, in mid-log phase, in a 1.0 ml total volume of spent media. Spent media refers to LB broth from the donor culture at a particular time point, which has been centrifuged to remove donor cells. For general mating assays, donor cultures were grown to approximately 0.5 OD_{600} and mated as above, in fresh LB broth. The mating cell mixtures were vortexed vigorously and placed on ice to halt further transfer. The cells were then serially diluted in SSC (150 mM NaCl, 15 mM Na-citrate, pH 7.0) and plated on the appropriate selective media. Donor cells containing pOX38-Tc or pOX38-Km were selected on LB agar containing either streptomycin and tetracycline, or streptomycin and kanamycin, respectively. Transconjugant cells containing pOX38-Tc or pOX38-Km were selected on LB agar containing either spectinomycin and tetracycline, or spectinomycin and kanamycin, respectively. Donor cells containing F*lac* or F*lac traJ90* were selected on LB agar containing streptomycin. Transconjugants containing F*lac* or F*lac traJ90* were selected on L1 agar containing spectinomycin. The plates were incubated for 1-2 days at 37°C and scored for growth.

Mating efficiency was measured as the percentage of transconjugants per donor. Each experiment was done at least three times.

2.4 Curvature prediction

Curvature predictions were done using the BEND-IT program (http://icgeb.org/dna/bend_it.html), which utilizes the BEND algorithm (Goodsell and Dickerson, 1994) to predict the degree of curvature per helical turn (10.5 bp). Fragments containing motifs with known intrinsic curvature generally give a result of 5° to 25° per helical turn (10.5 bp) using this algorithm, whereas those containing straight motifs are predicted to have under 5° curvature per helical turn.

2.5 Competitive H-NS electrophoretic mobility shift assays

Competitive EMSAs were performed based on a protocol previously described by Badaut *et al.*, (2002). A 473 bp target fragment containing P_M was generated by cutting pJLOY401 with BamHI and SalI resulting in a fragment stretching from *oriT* to the SalI site downstream of P_{M1}. A 355 bp target fragment containing P_J was generated by PCR using primers RWI65 and RWI68, resulting in a fragment stretching from the -202 position to the +153 position relative to the transcriptional start site at P_J. A 389 bp fragment of the P_Y region, stretching from the -260 to the +129 positions relative to the transcriptional start site at P_Y, containing the predicted bends was amplified via PCR using the oligonucleotides RWI79 and A2428. Approximately 100 ng of the indicated target DNA fragment was mixed in an equimolar ratio with pBR322, digested with TaqI and SspI for the P_J and P_Y assays, and TaqI, SspI, and NruI for the P_M assay. The reactions were incubated for 20 minutes at room temperature with increasing concentrations of pure H-NS (a gift from Dr. S. Rimsky, Universite Paris XI) in a 20 µl

reaction volume containing 40 mM Hepes, pH 8.0, 100 mM potassium glutamate, 10 mM magnesium aspartate, 0.1 mg/ml bovine serum albumin, 10% glycerol, 0.05% NP40 (Sigma), and 2 mM dithiothreitol. The DNA-protein complexes were then resolved on a 7.5% acrylamide/bisacrylamide (29:1) gel in TBE buffer at room temperature and stained with ethidium bromide.

2.6 DNase I footprinting assays

Approximately 50 pmol of RWI65 and RWI68 were 5' end-labeled with [γ-^{32}P-ATP] (NEN) using Polynucleotide Kinase (Roche Diagnostics) and the unincorporated label was removed using Oligo Quick Spin columns (Roche Diagnostics). The freshly labeled primer was used with 50 pmol of the unlabelled opposing primer to generate the substrate for footprint analysis of both the upper and lower strands by PCR amplification. The resulting product was electrophoresed on an 8% acrylamide/bisacrylamide (29:1) gel in TBE buffer, excised, and precipitated as described above. Approximately 20,000 cpm of the purified labeled product was used in each 20 µl footprinting reaction, containing 40 mM Hepes (pH 8.0), 8 mM magnesium aspartate, 45 mM potassium glutamate, 0.5 mM calcium chloride, 100 mg/ml bovine serum albumin, and 1 mM dithiothreitol. Each reaction was incubated with increasing concentrations of pure H-NS for 20 minutes at room temperature followed by the addition of 0.067 U of DNase I (GE Healthcare). The reactions were incubated for increasing amounts of time, depending on the amount of protein present, so as to maintain relatively constant cleavage of the DNA by the DNase I. Following a cleavage period of 2 to 5 minutes, the reaction was stopped by placing on ice and adding 150 µl of stop solution, containing 0.4 M sodium acetate (pH 5.2), 2.5 mM EDTA (pH 8.0), and 100 µg/ml sonicated calf thymus DNA. The reactions were

then extracted with 200 µl of phenol-chloroform and ethanol-precipitated before electrophoresis on a 6% polyacrylamide sequencing gel containing 6M urea (Maxam and Gilbert, 1980). The resulting fragments were sized by comparison to dideoxynucleotide sequencing reactions generated using the USB Thermosequenase Kit (GE Healthcare) and primers RWI65 and RWI68 for the upper and lower strand, respectively. Following electrophoresis, gels were dried and exposed on a Molecular Dynamics Storage Phosphor Screen. Gels were visualized on a Molecular Dynamics Phosphorimager 445 SI.

2.7 Immunoblot analysis

Unless otherwise stated, cultures were diluted 200-fold from standing overnight cultures into 25 ml fresh LB broth containing the appropriate antibiotics and grown under the indicated conditions. For growth rate analysis, overnight cultures were diluted 400-fold into 25 ml of either LB or MOPS medium. Culture samples equivalent to 0.1 OD_{600} were collected and pelleted at the indicated time points. These samples were boiled in sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970) for 5 minutes and then electrophoresed on a SDS 15% polyacrylamide gel. Following gel electrophoresis, samples were transferred onto Immobilon-P membranes (Millipore), in Towbin buffer (Towbin *et al.*, 1979). Membranes were then incubated overnight at 4° C in blocking solution containing 10% skim milk (Difco) dissolved in TBST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20). The blocked membranes were then incubated for one hour at room temperature in the same blocking solution containing diluted polyclonal antisera (anti-TraJ, 1:20,000; anti-TraM, 1:10,000; anti-TraY, 1:2000). Membranes were washed four times, for fifteen minutes each, in TBST at room temperature. Membranes were then incubated for 1 hour at room temperature in blocking

solution containing diluted secondary antibody (1:10,000 horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (GE Healthcare)), washed again as described above, and then developed using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and exposed to X-OMAT AR film (Kodak).

2.8 *In vitro* transcription

To generate FinP RNA for use as a probe for *traJ* mRNA and for Hfq binding studies, RNA was transcribed *in vitro* as previously described using pLJ5-13, which carries *finP* behind a T7 promoter, as a template (Jerome *et al.*, 1999). Briefly, pLJ5-13 was linearized by digesting with BamHI, which cuts the plasmid immediately downstream of *finP*, ensuring all transcripts are of a uniform length. Approximately 2 µg of template was used in a 20 µl reaction with 0.5 mM CTP, ATP, and GTP, and 0.02 mM UTP. Transcription reactions were performed for three hours at 37°C with 20 U of T7 RNA polymerase in the presence of 50 µCi [α-^{32}P-UTP] (3000 Ci/mmol; Perkin-Elmer), with 26 U of RNA Guard (GE Healthcare). The FinP has an additional G residue at the 5' terminus, which is necessary to facilitate transcription by the T7 RNA polymerase, and the sequence 5'-GGGGAUC-3' at the 3' terminus due to the presence of the BamHI restriction site. Completed reactions were incubated at 37°C for 15 minutes with DNase I (RNase-free) to remove any remaining template. RNA was then electrophoresed in a denaturing 8% TBE-polyacrylamide containing 8M urea and visualized with Kodak X-Omat film, cut out of the gel and eluted in DEPC-treated elution buffer (0.5 M ammonium acetate, 1 mM EDTA) overnight at 37°C. The eluent was then extracted with an equal volume of 1:1 phenol:chloroform, followed by an equal volume of chloroform. The RNA was precipated with 1 µl of glycogen (Roche), 0.1 volume sodium acetate, and

2 volumes of 95% ethanol. The pellet was washed twice with 70% ethanol, air-dryed, and dissolved in DEPC-treated H_2O. *traJ* RNA was similarly synthesized from a PCR-generated template, using the primer TvB15, which contains a functional T7 promoter, and the 3' primer, TvB14. One µl samples were collected before ("pre-cpm") and after ("post-cpm") gel purification and precipitation, subjected to scintillation counting, and used to quantify the yield of RNA with the following formula:

$$\text{Specific activity (S.A.)} = (\text{pre-cpm})(V_{total})/\text{pmol UTP in reaction}$$

$$\text{pmol RNA/µl} = (\text{post-cpm})/(\text{S.A.})(\text{number of U per RNA molecule})$$

2.9 Northern blot analysis

Cultures were diluted 200-fold from standing overnight cultures into 30 ml fresh LB broth containing the appropriate antibiotics. Culture samples equivalent to approximately 1.0 OD_{600} were collected from liquid cultures grown in LB at 37°C at the indicated time points and quickly pelleted and frozen in a dry ice-ethanol bath. For transcript half-life studies, rifampicin was added to the cultures to a final concentration of 200 µg/ml at 0 minutes. Total RNA was then isolated using the hot phenol method described previously (Jerome *et al.*, 1999). Briefly, cell pellets were resuspended in 300 µl of lysis buffer, containing 0.5% SDS (w/v), 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. Three hundred µl of phenol were added and the mixture was vortexed vigorously for approximately 30 seconds. The mixture was then incubated at 65°C for 10-15 minutes with regular vortexing for 5-10 seconds. The aqueous phase was separated following centrifugation at 13, 000 *g* for 15 minutes at 4°C and extracted twice with 300 µl of chloroform. RNA was precipitated with 30 µl 3 M sodium acetate and 2 volumes 95% ethanol, and washed twice with 70% ethanol to remove excess salt. RNA pellets

were dissolved in 20 µl of DEPC-treated H_2O and quantified by A_{260} using an Amersham Pharmacia Ultrospec 3000.

Samples containing 20 µg of total RNA were dissolved in 2x RNA loading dye (50% (v/v) deionized formamide, 5% (v/v) formaldehyde, 1x MOPS buffer (20 mM MOPS, pH 7.0; 50 mM sodium acetate; 1 mM EDTA), and 0.05% (w/v) bromophenol blue) and incubated at 65°C for 10 minutes. Samples were then electrophoresed on a 1.5% agarose gel containing 5% formaldehyde in MOPS buffer. Following electrophoresis, the RNA was transferred to a Zeta-Probe membrane (Bio-Rad) overnight in 20 x SSC (3 M sodium chloride; 0.3 M sodium citrate, pH 7.0). The membrane was then dried and cross-linked at 150 mJoules in a Bio-Rad GS Gene-linker. Blots probed for *traJ* were pre-hybridized at 58°C for 4 hours in hybridization buffer containing 50% (v/v) deionized formamide, 5x Denhardt's solution (20 mg/ml each of polyvinylpyrrolidone, Ficoll 400, and bovine serum albumin), 2.5x SSC, 1.5% (w/v) SDS, and 200 µg/ml each of freshly boiled *E. coli* strain W tRNA type XX (Sigma-Aldrich), and sonicated calf thymus DNA (Sigma-Aldrich). Blots were probed at 58°C overnight in fresh hybridization solution, containing 20 pmol of *in vitro* synthesized FinP RNA, universally labeled with [α-^{32}P-UTP]. Blots were washed at room temperature as follows: 5 minutes in 2x SSC, 10 minutes in 2x SSC/0.1% SDS, and10 minutes in 0.5x SSC/0.1% SDS. This was followed by a final wash in 0.1x SSC/0.1% SDS for 5 minutes at 55°C.

Blots to be probed for *traM* mRNA were prehybridized for 4 hours at 55° C in hybridization solution containing 2.5x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS, 90 mM Tris-HCl (pH 7.5), 0.9 M NaCl, 6 mM EDTA, 200 µg/ml of *E. coli* strain W tRNA

type XX (Sigma) and 200 μg/ml boiled, sonicated calf thymus DNA (Sigma). Blots were incubated overnight at 55°C in fresh oligonucleotide hybridization solution containing 20 pmol of SPE5-ext which was 5' end-labeled with [γ-^{32}P-ATP] (NEN) and T4 polynucleotide kinase (Roche Diagnostics), and purified on Quick Spin Oligo Columns (Roche Diagnostics). The blots were washed as described above. To probe for *traY* mRNA, [γ-^{32}P-ATP] end-labelled, purified RWI78 was used as a probe. All other steps were performed as described for *traM* mRNA. For the loading control, one of three protocols was used, as indicated. For the first protocol, the membrane was probed with 20 pmol of [γ-^{32}P-ATP] end-labeled LFR21, which binds non-specifically to 16S rRNA. All other steps were performed as described for *traM*, except that pre-hybridization and hybridization were done at 37°C. For the second protocol, the membrane was probed with 20 pmol of [γ-^{32}P-ATP] end-labelled 23SR3, which binds specifically to the 3' terminus of 23S rRNA. All other steps were performed as described for *traM* mRNA. For the third protocol, membranes were stained with Northern Blot Stain Blue (Sigma-Aldrich), which visibly stains rRNA, immediately after UV cross-linking. After washing, membranes were dried and then exposed on a Molecular Dynamics Storage Phosphor Screen and visualized on a Molecular Dynamics Phosphorimager 445 SI.

2.10 Primer extension analysis

Total cell RNA was isolated throughout the growth curve from MC4100/pOX38-Tc and PD32/pOX38-Tc as described in Section 2.9. Thirty μg of RNA were used for each reaction, with the oligonucleotide, SPE8, for analysis of P_M, which binds 108 bases downstream of P_{M1}, and RWI68, for analysis of P_J, which binds 134 bases downstream of the promoter. RNA samples were first digested with RNase-free DNase I (Amersham

Biosciences) for 1 hour at 37° C, then phenol-chloroform (1:1) extracted and ethanol-precipitated. RNA was mixed with 1 x 10^5 cpm of [γ-^{32}P-ATP] end-labeled oligonucleotide in a 30 µl reaction containing 1M NaCl, 160 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA, pH 8.0. Samples were incubated for 5 minutes at 85°C, then allowed to anneal at 37°C for 1 hour. Samples were precipitated and washed with 95% ethanol and redissolved in a 25 µl volume of AMV reverse transcriptase buffer, containing 0.5 mM dNTPs and 15 units of RNAguard (GE Healthcare). Fifteen units of AMV reverse transcriptase (Roche Diagnostics) were added to each reaction, which were then incubated for 1 hour at 42°C. Remaining RNA was removed by digestion with RNase A for 15 minutes at 37°C. DNA was then ethanol-precipitated and separated by electrophoresis in a 6% polyacrylamide sequencing gel (Maxam and Gilbert, 1980). Dideoxynucleotide sequencing reactions were performed using the USB Thermosequenase kit (GE Healthcare) and the same primers as were used for the primer extension reactions, and run alongside the samples as a standard. Gels were then dried and exposed on a Molecular Dynamics Storage Phosphor Screen and visualized on a Molecular Dynamics Phosphorimager 445 SI.

2.11 β-galactosidase assays

The activity of *lacZ* fusions to the *traJ* and *traM* promoters was assayed using constructs described in Table 1. All assays were performed as described by Miller (1972), and the results were reported in Miller Units (MU). Activity in MU is determined using the formula MU = 1000 (A_{420}/(tvOD_{600})), where t = reaction time (in minutes) and v = culture volume added (in ml). Strains containing the appropriate fusion constructs were diluted from standing overnight cultures into fresh LB broth containing

chloramphenicol. MC4100 was diluted 1:200, whereas PD32 was diluted 1:100 to compensate for the slow emergence from stationary phase and growth rate. Experiments were performed at least three times and the average and standard deviation of the results was determined.

2.12 Overexpression and purification of Hfq

Hfq was overexpressed and purified as a 6x-His fusion protein from pTE607 (kindly provided by T. Elliott) in BL21(DE3) pLysS as described by Folichon *et al.*, (2003) with minor modifications. Briefly, a 500 ml culture was grown at 37°C in LB broth to approximately 0.5 OD_{600} and then induced with 1 mM IPTG for 2.5 hours. Cultures were then pelleted, and the pellets were stored at -80°C until processing. Pellets were re-suspended in chilled lysis buffer, containing 20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 10% glycerol (v/v), and 0.1% Triton X-100. The suspension was then passed through a French press, and the lysate was cleared by centrifugation for 30 minutes at 15, 000 *g* and 4°C. Imidazole-HCl (pH 7.8) was added to a final concentration of 1 mM and the lysate was incubated with 1 ml of Ni^{2+}-NTA agarose with gentle agitation at 4°C for 1 hour. Following incubation, the slurry was applied to a column and washed with approximately 15 ml of wash buffer I, containing 20 mM Tris-HCl (pH 7.8), 300 mM NaCl, and 20 mM imidazole. The column was then washed with 15 ml of wash buffer II, containing 50 mM sodium phosphate (pH 6.0) and 300 mM NaCl. Protein was eluted from the column with buffer containing 50 mM sodium phosphate (pH 6.0), 300 mM NaCl, and 250 mM imidazole. Protein fractions containing Hfq were identified by SDS-PAGE, pooled, and incubated at 80°C for 15 minutes. Insoluble contaminants were removed immediately by centrifugation at 7000 *g* for 5 minutes at room temperature.

Purified protein was dialyzed against storage buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NH_4Cl, 20% glycerol (v/v), and 0.1% Triton X-100 (v/v). Pure Hfq was quantified using a standard curve of bovine serum albumin via the Bradford protein assay and stored at -20°C.

2.13 Hfq electrophoretic mobility shift assays

RNA binding assays were performed in 50 µl of reaction buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 80 mM NaCl, 10% glycerol (v/v), and 0.01% dodecyl maltoside (v/v). Five fmol of *in vitro* transcribed [^{32}P]-labeled RNA was incubated in the reaction with increasing concentrations of Hfq for 20 minutes at 37°C. Competitive binding assays were performed in the presence of 100 ng/µl *E. coli* tRNA. Following the incubation, reactions were loaded onto native 6% acrylamide gels run in 1 × TBE at 4°C. Gels were then dried, exposed to a Molecular Dynamics Storage Phosphor Screen overnight, developed on a Molecular Dynamics Phosphor Imager 445 SI and analyzed using ImageQuaNT software (GE Healthcare). A dissociation constant (K_d) was determined from the Hfq concentration that caused 50% of the labeled RNA to shift in the gel using the following formula:

$$K_d = [\text{Hfq}][\text{RNA}]/[\text{Hfq-RNA}]$$

At the point of 50% binding, the concentration of free RNA [RNA] and the Hfq-RNA complex [Hfq-RNA], should be equal, and the K_d effectively becomes the concentration of free Hfq [Hfq], as Hfq is present in molar excess to the RNA target. All bound RNA was considered a single species.

2.14 Isoelectric focusing analysis

Overnight cultures were diluted 1:200 into 20 ml fresh LB broth and incubated at 37°C with shaking. At the indicated time points, volumes equivalent to approximately 8 OD_{600} were removed and pelleted. Cell pellets were then resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mg/ml lysozyme, and Complete Mini (EDTA-free) protease inhibitor (Roche Diagnostics). The suspension was incubated at 4°C for 30 minutes on a roller and then lysed with a sonicator. Lysates were cleared twice by centrifugation at 16 000 *g* for 15 minutes at 4°C and protein concentrations were determined by A_{280} on an Amersham Pharmacia Ultrospec 3000. Forty µg protein samples were then loaded in 10% glycerol and electrophoresed in a Ready Gel IEF Gel (Bio-Rad). Following electrophoresis, gels were transferred to Immobilon-P membrane (Millipore) in 0.7% acetic acid at 100 V for 1 hr. All blocking, probing, washing, and visualization steps were performed as described for standard immunoblot analysis.

2.15 Fis and IHF electrophoretic mobility shift assays

The specific binding activity of IHF and Fis at P_Y was examined using electrophoretic mobility shift assays. A 325-bp, universally labeled P_Y fragment was generated via PCR by including approximately 50 µCi [α-^{32}P]-dCTP (Amersham Phamacia) in the PCR reaction with the primers A2426 and A2428. Fragments were purified as described in Section 2.2. The radioactivity of 1 µl samples collected from the reaction before ("pre-cpm") and after ("post-cpm") gel-purification and precipitation was quantified in a liquid scintillation counter and used to quantify DNA yield using the following formula:

$$\text{Specific activity (S.A.)} = (\text{pre-cpm})(V_{total})/\text{pmol CTP in reaction}$$

$$\text{pmol DNA/µl} = (\text{post-cpm})/(\text{S.A.})(\text{number of C/PCR product})$$

For Fis, binding reactions (15 µl) were set up on ice, containing 10 fmol labeled DNA and varying amounts of purified Fis protein (a gift from R.L. Gourse, University of Wisconsin), and binding buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1mM DTT, 100 mM NaCl, 10% glycerol, and 25 µg/ml poly dI-dC), and incubated for 10 minutes at 22°C. Samples were loaded onto a running 8% polyacrylamide:-*bis*-acrylamide (29:1) gel in TBE buffer. The gel was run at 40 mA at 4° C, dried, exposed to a Molecular Dynamics Storage Phosphor Screen overnight, which was then scanned using a Molecular Dynamics Phosphor Imager 445 SI.

For IHF, binding reactions (10 µl) were set up on ice, containing 10 fmol labeled DNA, and varying amounts of purified IHF protein (a gift from S.D. Goodman, University of Southern California), in binding buffer (50 mM Tris-HCl, pH 7.5, 70 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 100 µg/ml bovine serum albumin, 10% glycerol, and 25 µg/ml poly dI-dC) and incubated for 10 minutes at 22°C. Reactions were then loaded, electrophoresed, and visualized as described above for Fis.

Chapter 3: The role of H-NS in silencing F transfer gene expression during entry into stationary phase*

*Portions of this chapter were published: Will, W.R, Lu, J., and Frost, L.S. (2004) *Mol Microbiol* **54**: 769-782.

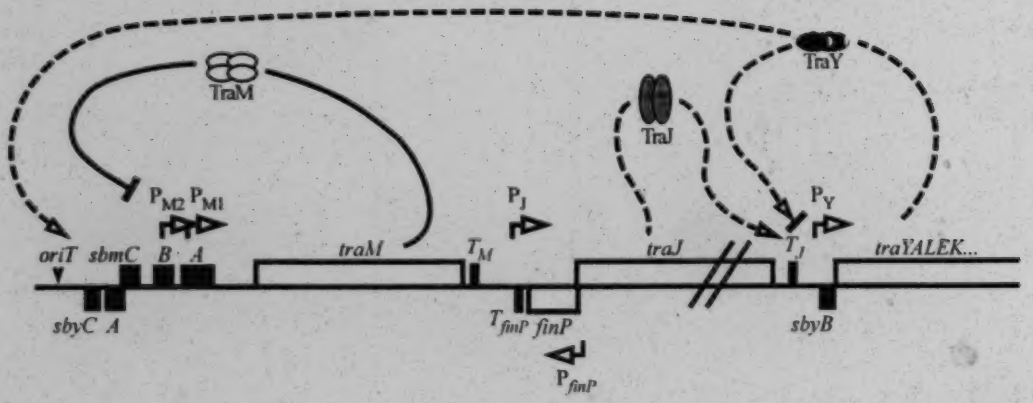
3.1 Introduction

The F plasmid is considered to be a paradigm of bacterial conjugation in *Escherichia coli* (Lawley *et al.*, 2004). The primary regulatory circuit of F transfer involves three plasmid-encoded proteins: TraJ, TraM, and TraY (Frost *et al.*, 1994). TraJ is expressed from its own monocistronic operon and is thought to be the primary activator of transfer gene expression (Fig. 3.1A) (Willetts, 1977). In most F-like plasmids, *traJ* is repressed by an antisense RNA, *finP*, in conjunction with a chaperone, FinO (van Biesen and Frost, 1994; Arthur *et al.*, 2003). In F, *finO* is interrupted by an IS*3* element, resulting in the derepression of both *traJ* expression and F plasmid transfer (Cheah and Skurray, 1986). TraJ upregulates the 33.3 kb polycistronic transfer (*tra*) operon, which contains all of the plasmid genes necessary for transfer, save *traJ* and *traM* (Silverman *et al.*, 1991a). The activation of the *tra* operon results in an increase in TraY, which is thought to further activate its own transcription (Silverman and Sholl, 1996), although conflicting evidence suggests that TraY is also an autorepressor (Taki *et al.*, 1998). TraY is an essential component of the relaxosome, a nucleosomal complex forming at the origin of transfer, which processes F plasmid DNA for transfer. TraY also activates the expression of the monocistronic *traM* operon (Penfold *et al.*, 1996). Finally, TraM acts as an autoregulator, repressing its own expression. TraM is also thought to act as a signalling protein between the relaxosome and the transferosome, the multi-component protein complex responsible for actually transporting the DNA across the donor cell membrane and into the recipient (Disque-Kochem and Dreiseikelmann, 1997).

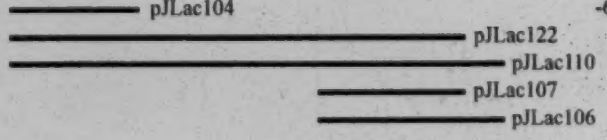
F transfer is extremely growth phase-dependent, since transfer efficiency drops precipitously as donor cells enter stationary phase (Frost and Manchak, 1998). The

Figure 3.1 A model for the regulatory circuit of F plasmid transfer gene expression (A). Positive effects are indicated by dashed lines ending in arrowheads. Negative effects are indicated by solid lines ending in bars. As TraY appears to act as both a repressor and an activator at P_Y, the effect is indicated by a dashed line ending in both an arrowhead and a bar. P_{M1} and P_{M2} indicate *traM*' promoters, while P_J, P_{finP}, and P_Y indicate the *traJ*, *finP*, and *traY* promoters, respectively. T_M, T_{finP}, and T_J denote the *traM*, *finP*, and *traJ* transcriptional terminators, respectively. *sbmA, B,* and *C* are TraM binding sites, while *sbyA*, *B*, and *C* are TraY binding sites. The relative positions of all F plasmid regulatory protein binding sites are indicated as previously reported (Frost, *et al.*, 1994). (B) The effect of H-NS on individual *tra* promoters was examined using a series of *lacZ* transcriptional fusions constructed in the low-copy transcriptional fusion vector, pJLac101. The relative size and content of each fusion construct is indicated by solid lines beneath the map. The length of each promoter fragment is indicated relative to P_J. In the case of pJLac104, the P_M transcriptional fusion, the coordinates in parentheses are relative to P_{M1}. For each construct, standing overnight cultures of both MC4100 and PD32 were diluted into fresh LB broth, and β-galactosidase assays were performed after three and seven hours of growth. Results are given in Miller Units (MU) (Miller, 1972), and indicate the average and standard deviation of three separate experiments.

A)



B)



	Coordinates	3 hours		7 hours	
		MC4100	PD32	MC4100	PD32
pJLac104	-696(-138) / -504(+54)	7357 +/- 1067	5928 +/- 1502	10167 +/- 2014	12138 +/- 1561
pJLac122	-696 / +118	5752 +/- 400	2491 +/- 164	16004 +/- 1969	4715 +/- 342
pJLac110	-696 / +153	1203 +/- 260	2339 +/- 188	2861 +/- 376	5557 +/- 144
pJLac107	-141 / +118	4578 +/- 1143	1759 +/- 669	10276 +/- 2834	3124 +/- 448
pJLac106	-141 / +153	3846 +/- 317	2399 +/- 188	4852 +/- 132	4354 +/- 56

factors responsible for this decline in mating ability are unknown, but appear to provide sensitivity to the physiological state of the host. Numerous host factors are known to influence transfer. Integration host factor (IHF) has been shown to be essential for formation of the relaxosome (Nelson *et al.*, 1995), and may also play a role in *tra* operon control (Gamas *et al.*, 1987; Silverman *et al.*, 1991a). ArcA (SfrA) has been shown to bind upstream of the *tra* operon promoter, P_Y, activating transcription in conjunction with TraJ (Silverman *et al.*, 1991; Strohmaier *et al* 1998). The expression of *traJ* in F-like plasmids has been shown to be regulated by numerous factors, including Lrp and CRP (Camacho and Casadesus, 2002; Starcic *et al.*, 2003), whereas the stability of the TraJ protein is influenced by the Cpx extracytoplasmic stress response (Gubbins *et al.*, 2002).

Numerous other systems in *E. coli* that exhibit growth phase-dependent regulation have been shown to be regulated by the nucleoid associated protein, H-NS (Hommais *et al.*, 2001). H-NS is a 15.4 kDa cytoplasmic protein that binds preferentially to segments of intrinsically curved DNA at sub-saturating cellular concentrations (Pon *et al.*, 1988; Owen-Hughes *et al.*, 1992). However, at high concentrations *in vitro*, H-NS is capable of binding non-specifically to extended segments of DNA. Because of this, H-NS is generally thought to act as a transcriptional silencer that binds preferentially to a region of curved DNA, and then proceeds to nucleate along the DNA, thereby repressing any nearby promoters (Williams and Rimsky, 1997; Rimsky *et al.*, 2001). H-NS has also been shown to play a significant role in structuring and organizing the bacterial chromosome, possessing the ability to alter supercoiling and condense DNA (Dame *et al.*, 2000). Intracellular H-NS levels appear to be relatively constitutive throughout growth, at a level of approximately 20,000 molecules per cell, with *hns* transcription being

coupled to chromosomal DNA synthesis (Free and Dorman, 1995; Williams and Rimsky, 1997). Although instances of transcriptional activation directly mediated by H-NS are relatively uncommon (Hommais *et al.*, 2001), H-NS was recently shown to act as an activator of *traJ* expression in the F-like plasmid pRK100 (Starcic-Erjavec *et al.*, 2003). This study provides evidence that in the case of the F plasmid, H-NS acts as a potent repressor of *traJ* and *traM* transcription. Furthermore, this H-NS-mediated repression appears to be pivotal in down-regulating F transfer as the host cell enters stationary phase.

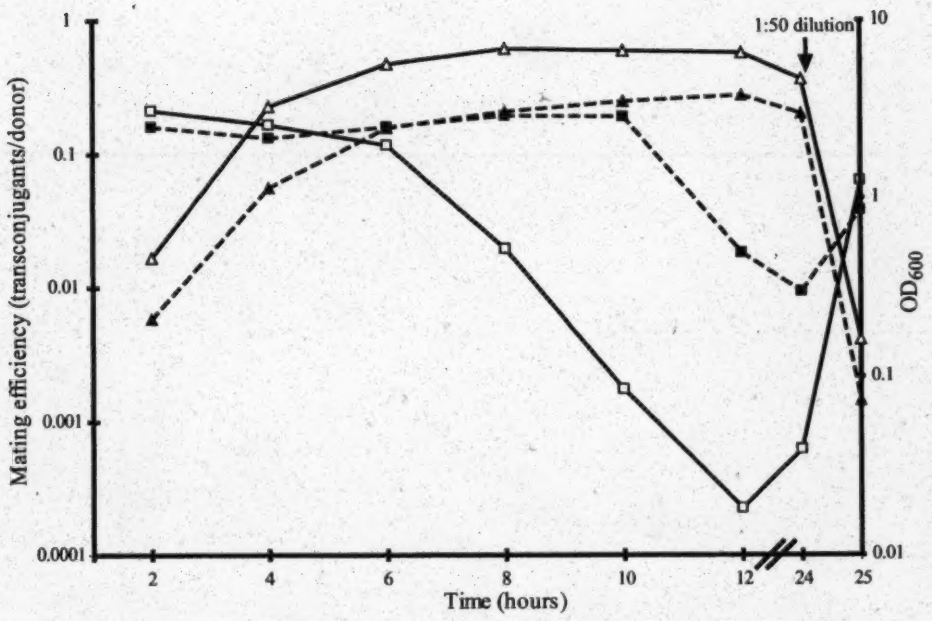
3.2 Results

3.2.1 Transfer efficiency and growth is altered in an *hns* mutant

The effect of an *hns* mutation on F transfer throughout the growth cycle was examined using mating assays with wild-type MC4100 and the *hns* mutant, PD32, containing pOX38-Tc, an F plasmid derivative containing the entire F transfer region. The results suggested that H-NS down-regulates transfer ability as the culture progresses through the growth phase (Fig. 3.2), since PD32/pOX38-Tc displayed prolonged mating in stationary phase. Mating efficiency of PD32/pOX38-Tc began to decrease after approximately 10 to 12 hours, compared to MC4100/pOX38-Tc that began to decrease after 6 to 8 hours. After 24 hours of growth, transfer efficiency was approximately 10-fold higher in the *hns* mutant than in the wild-type donor cells. Upon dilution of the donor cultures into fresh LB broth, transfer ability was quickly re-established in both strains.

PD32/pOX38-Tc grew more slowly when compared to MC4100/pOX38-Tc (Fig. 3.2). Whereas a slow growth phenotype is generally common to *hns* mutant strains

Figure 3.2 F plasmid transfer is prolonged in an *hns* mutant. Overnight cultures of MC4100 and PD32 containing pOX38-Tc were diluted 200-fold in fresh LB broth, and incubated with shaking at 37° C. At the indicated time points, a volume of cells equivalent to 0.1 OD_{600} was mated with ED24 in spent media, serially diluted, and plated on media selective for transconjugants. After 24 hours, donor cultures were diluted 50-fold in fresh LB broth, and were examined for their ability to re-establish mating. Mating efficiency is indicated by squares, whereas OD_{600} is indicated by triangles. Data for MC4100 is indicated by a solid line, whereas data for PD32 is indicated by a dashed line.

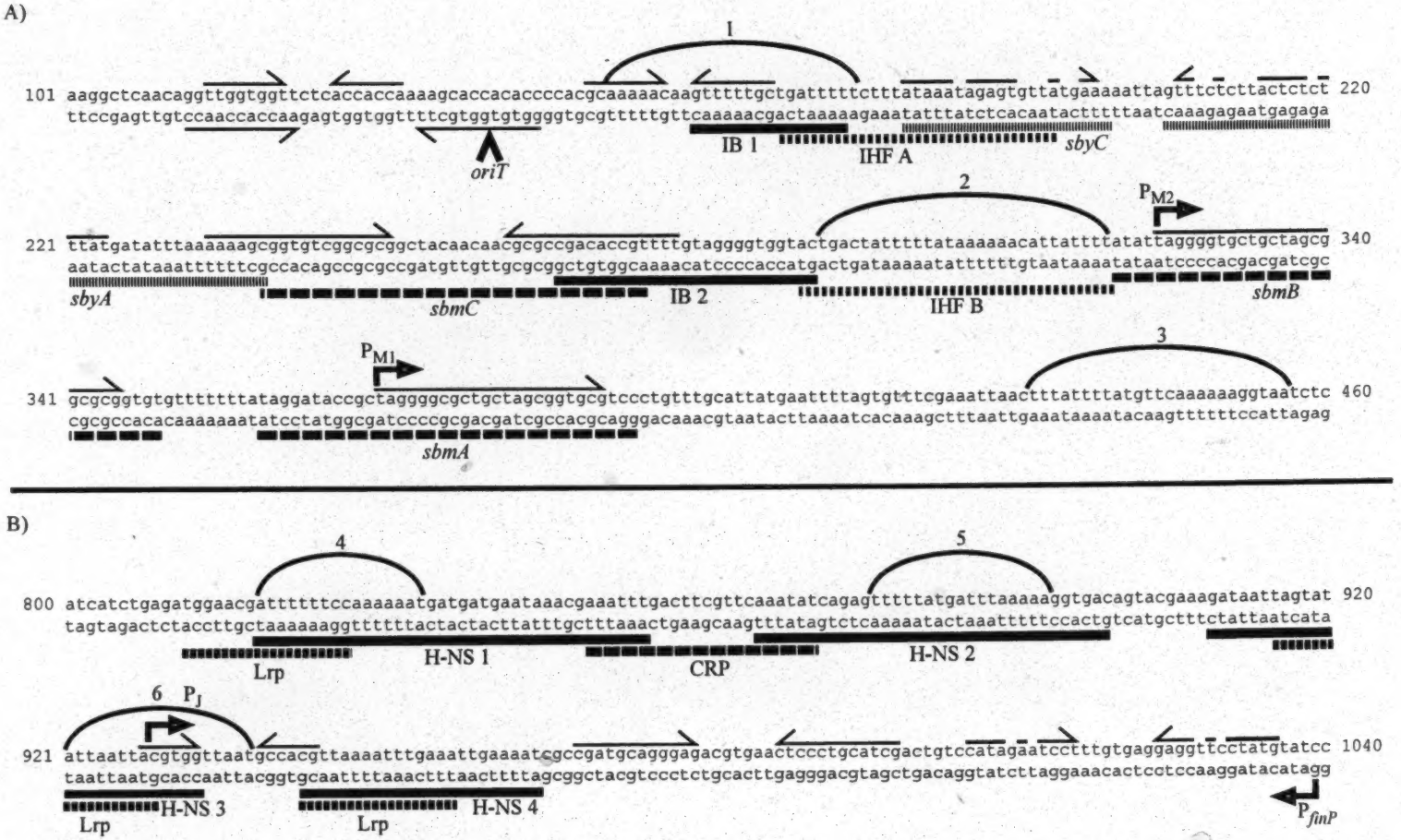

1:50 dilution
Mating efficiency (transconjugants/donor)
1
0.1
0.01
0.001
0.0001
10
1
0.1
0.01
OD600
2
4
6
8
10
12
24
25
Time (hours)

(Barth *et al.*, 1995; Yamashino *et al.*, 1995), PD32/pOX38-Tc grew somewhat more slowly than PD32 alone (data not shown) and reached a density of approximately 50% that of wild-type donors or PD32 alone. Liquid cultures of PD32/pOX38-Tc also appeared grainy relative to both MC4100/pOX38Tc and PD32, with cells aggregated into large clumps that disaggregated upon vortexing (data not shown). This tendency to aggregate could artificially lower the apparent cell density. The observed graininess results from hyper-piliation, suggesting that F pili synthesis was up-regulated in the *hns* mutant.

3.2.2 DNA curvature prediction at *traM* and *traJ*

H-NS does not possess a strong consensus binding sequence, but rather binds preferentially to structurally distinct segments of DNA containing intrinsic curves (Owen-Hughes *et al.*, 1992). The 2 kb region of the F plasmid containing the three transfer regulatory genes, *traM*, *traJ*, and *traY*, and their promoters, was examined for regions of significant curvature using the BEND-IT computer program (http://icgeb.org/dna/bend_it.html). Whereas segments of DNA containing known curved motifs have been predicted to have 5°- 25° of curvature per helical turn using the BEND algorithm (Goodsell and Dickerson, 1994), segments of DNA with a minimum predicted curvature of approximately 10° per helical turn were selected with the expectation that these segments would promote H-NS binding *in vivo*. Analysis of the *traM* promoter region revealed three regions of significant curvature (curves 1, 2, and 3; Fig. 3.3A) that were dispersed throughout the region, at approximately 100 bp intervals. Interestingly, curves 1 and 2 at least partially overlap IHF binding sites in the *oriT* region (Frost *et al.*, 1994). Whether this is indicative of competition between IHF and H-NS for these sites is

Figure 3.3 The *traM* and *traJ* promoter regions contain regions of predicted curvature. The sequences surrounding the *traM* promoters, P_{M1} and P_{M2} (A), and the *traJ* promoter, P_J (B), were analyzed for the presence of intrinsic curvature using the BEND-IT computer program. Regions of DNA predicted to have significant intrinsic curvature (approximately 10° per helical turn) are indicated by a curved line immediately above the sequence. DNA-binding protein binding sites are indicated by lines immediately beneath the sequence. The indicated H-NS binding sites correspond to regions of H-NS-mediated DNase I protection. The indicated Lrp and CRP binding sites are based on studies in related systems (Camacho and Casadesus, 2002; Starcic *et al.*, 2003; Starcic-Erjavec *et al.*, 2003). All other features are shown as previously reported (Frost *et al.*, 1994). *sbyA* and *sbyB* are TraY binding sites, whereas *sbmA*, *B* and *C* are TraM binding sites. IHF binding sites are designated IHF A and B. IB1 and IB2 are regions of intrinsic bending identified by previous studies (Tsai, Fu, Deonier, 1990). Sequence numbering indicates its position relative to the BglII site at the start of the transfer region sequence (Frost *et al.*, 1994).


A)
1
101 aaggctcaacaggttggtggttctcaccaccaaaagcaccacaccccacgcaaaaacaagtttttgctgatttttctttataaatagagtgttatgaaaaattagtttctcttactctct 220
ttccgagttgtccaaccaccaagagtggtggttttcgtggtgtggggtgcgtttttgttcaaaaacgactaaaaagaaatatttatctcacaatacttttttaatcaaagagaatgagaga
oriT
IB 1
IHF A
sbyC
2
P M2
221 ttatgatatttaaaaaagcggtgtcggcgcggctacaacaacgcgccgacaccgttttgtaggggtggtactgactatttttataaaaaacattatttttatattaggggtgctgctagcg 340
aatactataaatttttttcgccacagccgcgccgatgttgttgcgcggctgtggcaaaacatccccaccatgactgatataaaaatatttttttgtaataaaatataatccccacgacgatcgc
sbyA
sbmC
IB 2
IHF B
sbmB
3
P M1
341 gcgcggtgtgtttttttataggataccgctaggggcgctgctagcggtgcgtccctgtttgcattatgaattttagtgtttcgaaattaactttatttttatgttcaaaaaaggtaatctc 460
cgcgccacacaaaaaaatatcctatggcgatccccgcgacgatcgccacgcagggacaaacgtaatacttaaaatcacaaagctttaattgaaataaaatacaagttttttccattagag
sbmA
B)
4
5
800 atcatctgagatggaacgatttttttccaaaaaatgatgatgaataaacgaaatttgacttcgttcaaatatcagagtttttatgatttaaaaaggtgacagtacgaaagataattagtat 920
tagtagactctaccttgctaaaaaaggtttttttactactacttatttgctttaaactgaagcaagtttatagtctcaaaaatactaaatttttccactgtcatgctttctattaatcata
Lrp
H-NS 1
CRP
H-NS 2
6 P J
921 attaattacgtggttaatgccacgttaaaatttgaaattgaaaatcgccgatgcagggagacgtgaactccctgcatcgactgtccatagaatcctttgtgaggaggttcctatgtatcc 1040
taattaatgcaccaattacggtgcaattttaaactttaacttttagcggctacgtccctctgcacttgagggacgtagctgacaggtatcttaggaaacactcctccaaggatacatagg
Lrp
H-NS 3
Lrp
H-NS 4
P finP

unknown. This curvature may simply be reflective of the requirement of the DNA in an IHF-DNA complex to bend to an angle of over 160° (Rice *et al.*, 1996).

The region upstream of the *traJ* promoter contains three segments with significant predicted curvature (curves 4, 5, and 6), tightly grouped together within a region of approximately 125 bp (Fig. 3.3B), which is a strong candidate for H-NS binding. Interestingly, curves 4 and 5 flank the putative binding sites for Lrp and CRP, previously identified to be activators of *traJ* in the F-like plasmids, pSLT and pRK100 (Camacho and Casadesus, 2002; Starcic *et al.*, 2003; Starcic-Erjavec *et al.*, 2003). Previous studies have also identified curve 5 in pRK100 as a potential H-NS binding site (Starcic-Erjavec *et al.*, 2003). Curve 6 also overlaps a predicted Lrp binding site, suggesting that Lrp may interact with H-NS (Starcic-Erjavec *et al.*, 2003).

Although the region immediately surrounding P_Y did show predicted intrinsic curvature (data not shown), the predicted curves were not as strong as those observed at P_J and P_M. This region will be discussed in Chapter 4.

3.2.3 H-NS binds to the P_M and P_J regions

Competitive electrophoretic mobility shift assays were performed to determine if the predicted curved regions near the promoters for *traM* and *traJ* could provide a target for preferential H-NS binding. DNA fragments containing either promoter were mixed with equimolar amounts of restriction endonuclease-digested pBR322 and incubated in the presence of pure native H-NS. pBR322 contains a copy of the *bla* gene, which has been shown to be a preferential target for H-NS binding (Zuber *et al.*, 1994) and was used as a competitor in the H-NS binding assays. H-NS appeared to bind P_J with slightly greater affinity than P_M, which in turn demonstrated an affinity that was equal to or

greater than that of the pBR322 competitor fragments, including the 215 bp *bla* fragment containing an H-NS binding site (Fig. 3.4), indicating specific binding to the P_M and P_J regions.

3.2.4 DNase I footprinting of the P_J promoter

DNase I footprinting analysis was performed on the upper and lower strands of DNA fragments containing the P_J promoter to identify the precise locations of H-NS binding. The higher affinity of H-NS for P_J, as well as the tight grouping of the predicted curves when compared to P_M, made P_J a good target for footprinting analysis. Footprinting assays on the upper strand revealed three regions of H-NS-mediated DNase I protection (Fig. 3.5A). These regions, designated H-NS 1, H-NS 2, and H-NS 3, overlap predicted curves 4, 5, and 6 respectively (Fig. 3.3B). Analysis of the lower strand identified four regions of H-NS-mediated DNase I protection (Fig. 3.5B). These corresponded to the three footprints seen on the upper strand, as well as H-NS 4, which is positioned immediately downstream of curve 6. Given that the sequence of H-NS 4 displays a regular phasing of poly-A poly-T DNA, (Fig. 3.3B) it might possess curvature not accurately predicted by the BEND-IT program. Nonetheless, these results show that H-NS binds extensively to a 150 bp region at P_J including all of the regions containing predicted curvature.

3.2.5 TraJ and TraM levels are increased in *hns* cells

The hyper-piliated phenotype of *hns* mutant hosts suggested that *tra* gene expression was derepressed. To test this hypothesis, immunoblots to detect TraM, TraJ and TraY were performed on whole cell lysates from samples collected throughout the

Figure 3.4 H-NS binds preferentially to the *traJ* and *traM* promoter regions. Pure H-NS was incubated with equimolar amounts of restriction endonuclease-digested pBR322 and promoter fragment containing the regions of predicted curvature. The DNA fragments were then resolved by electrophoresis on a 7.5% acrylamide gel. The *traJ* promoter region (A) was mixed with TaqI-SspI digested pBR322, whereas the *traM* promoter region (B) was mixed with TaqI-SspI-NruI digested pBR322, which was necessary to resolve a single pBR322 fragment that migrates at the same position as the *traM* promoter target fragment. The concentrations of H-NS used in each reaction are indicated above the gel. The 215 bp *bla* promoter fragment to which H-NS preferentially binds is also indicated.

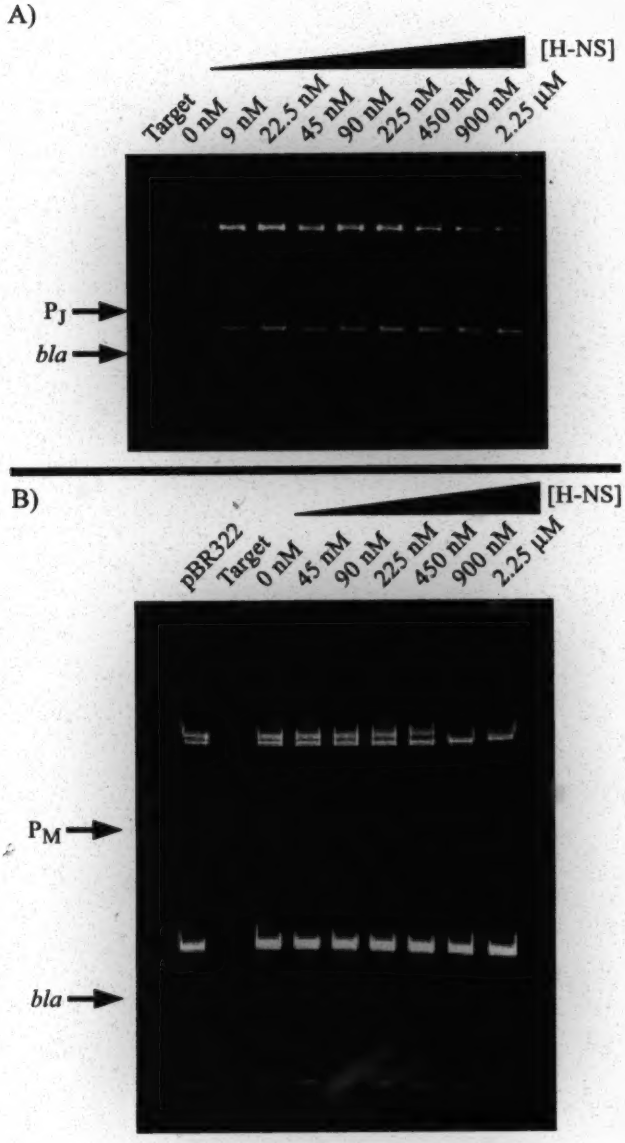
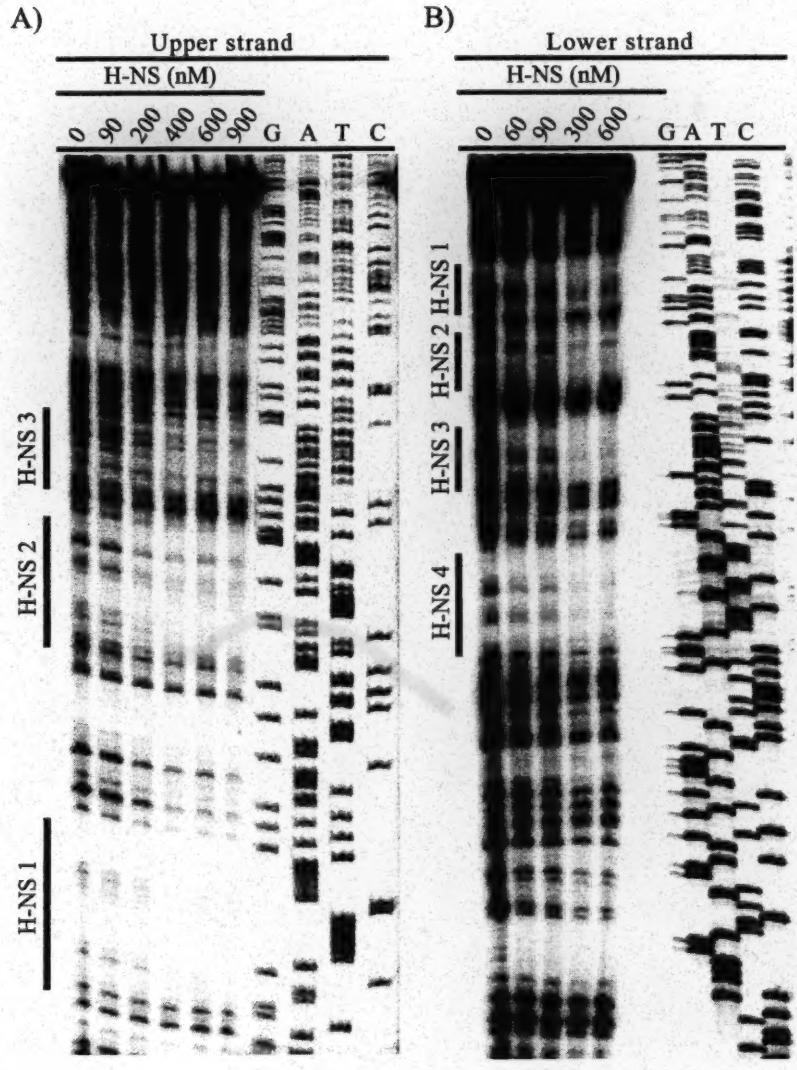

A)
[H-NS]
Target
0 nM
9 nM
22.5 nM
45 nM
90 nM
225 nM
450 nM
900 nM
2.25 µM
P_J
bla
B)
[H-NS]
pBR322
Target
0 nM
45 nM
90 nM
225 nM
450 nM
900 nM
2.25 µM
P_M
bla

Figure 3.5 Analysis of H-NS binding at the *traJ* promoter by DNase I footprinting. DNase I footprinting analysis was performed on both the upper (A) and lower (B) strands of the *traJ* promoter region, using a 355 bp fragment generated by the oligonucleotides RWI65 and RWI68 incubated with increasing concentrations of H-NS. The concentrations of H-NS used in each reaction are indicated above each lane. Sequencing ladder standards, indicated by G, A, T, and C, are shown at the right of each footprint. Solid bars indicate regions of H-NS-mediated DNase I protection, which have been designated H-NS 1, H-NS 2, H-NS 3, and H-NS 4.


A)
Upper strand
H-NS (nM)
0 90 200 400 600 900 G A T C
H-NS 1
H-NS 2
H-NS 3
B)
Lower strand
H-NS (nM)
0 60 90 300 600 G A T C
H-NS 1
H-NS 2
H-NS 3
H-NS 4

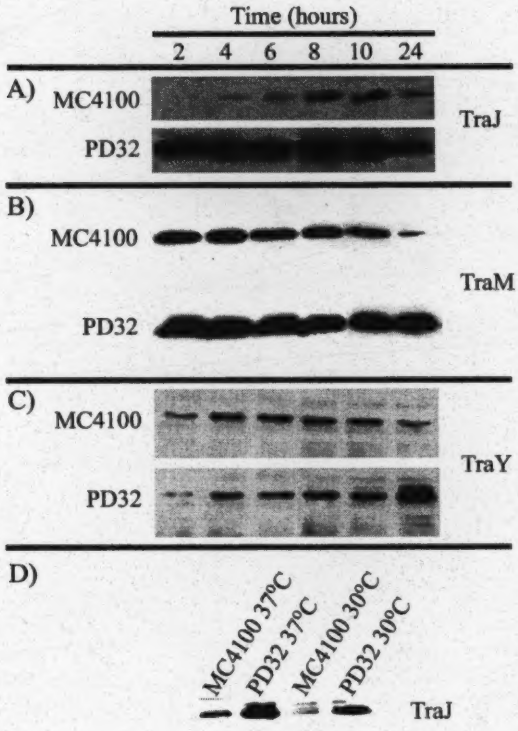
growth curve from MC4100/pOX38-Tc and PD32/pOX38-Tc cultures. TraJ levels were greatly increased throughout the growth curve in PD32/pOX38-Tc compared to MC4100/pOX38-Tc, even after 24 hours of growth (Fig. 3.6A). Western analysis of TraM gave similar results to TraJ (Fig. 3.6B). TraM levels appeared to increase in PD32/pOX38-Tc relative to MC4100/pOX38-Tc, particularly in stationary phase. However, the difference does not appear to be as significant as was seen in the case of TraJ. The increase in TraM could be an indirect effect of the increase in TraJ, which increases levels of TraY, the *traM* transcriptional activator. TraY levels exhibited the least change of the three proteins, with slightly elevated levels in PD32/pOX38-Tc in late stationary phase (Fig. 3.6C).

As H-NS regulates *Shigella flexneri virF* in a temperature dependent manner (Prosseda *et al.*, 2004), it seemed possible that H-NS was also responsible for thermosensitivity of F plasmid transfer. To determine if H-NS regulates transfer in a temperature-dependent manner, TraJ levels were assayed in exponential-phase MC4100/pOX38-Tc and PD32/pOX38-Tc cultures grown at 37°C and 30°C (Fig. 3.6D). Whereas TraJ levels decreased in MC4100/pOX38-Tc when the growth temperature was decreased to 30°C, it was unaffected in PD32/pOX38-Tc, suggesting that H-NS also regulates plasmid transfer in a temperature-dependent manner.

3.2.6 Analysis of *traM* and *traJ* mRNA levels in *hns* cells

In order to confirm that H-NS controls *traJ* and *traM* at the transcriptional level, and not indirectly at the post-transcriptional level, Northern blot analyses were performed on total cellular RNA samples taken at 3, 5, 7, and 9 hours of growth from both MC4100/pOX38-Tc and PD32/pOX38-Tc donor cultures. RNA was electrophoresed on

Figure 3.6 Immunoblot analysis of TraM, TraJ, and TraY in an *hns* mutant. Standing overnight cultures of MC4100/pOX38-Tc and PD32/pOX38-Tc were diluted 200-fold in fresh LB broth and incubated at 37° C with shaking. At the indicated time-points, culture samples equivalent to 0.1 OD_{600} were collected, pelleted, and probed via immunoblot analysis for levels of TraJ (A), TraM (B), and TraY (C). As TraJ levels were the most strongly affected, TraJ levels in exponential phase cultures were examined for H-NS-mediated thermoregulation (D). Cultures were inoculated as indicated above, grown at either 37°C or 30°C to exponential phase and cell pellets were collected for immunoblot analysis.


Time (hours)
2 4 6 8 10 24
A)
MC4100
PD32
TraJ
B)
MC4100
PD32
TraM
C)
MC4100
PD32
TraY
D)
MC4100 37°C
PD32 37°C
MC4100 30°C
PD32 30°C
TraJ

a formaldehyde-agarose gel, transferred to a nylon membrane, and then probed with [^{32}P]-labelled *finP* RNA synthesized *in vitro* to detect *traJ* transcripts. Whereas *traJ* mRNA was extremely scarce in MC4100/pOX38-Tc, particularly at time points beyond three hours, transcript levels were elevated in all samples from PD32/pOX38-Tc, peaking between five and seven hours (Fig. 3.7A). Also of note is the number of transcripts detected which are longer than *traJ*, which is approximately 0.9 kb in size (Lee *et al.*, 1992). Major transcripts of approximately 2.4 kb, 2 kb, 1.4 kb, and 0.9 kb in size, as well as smaller degradation products, which appear at later time-points, were detected. Therefore, either transcription from P_J is reading through into the *tra* operon downstream, or the P_M promoters are reading through into *traJ*. Primer extension analysis of total cellular RNA at the same time points identified a single major transcript originating from P_J (Fig. 3.8A). In MC4100/pOX38-Tc, this transcript decreased at later time points, however the transcript was visible throughout the growth cycle in PD32/pOX38-Tc. The additional, smaller fragments detectable in PD32/pOX38-Tc are either degradation products or artifacts caused by the stalling of the reverse transcriptase on the extensive secondary structure downstream of P_J in the 5' UTR of *traJ*.

The presence of *traM* transcripts was also detected by northern blot analysis using the [^{32}P]-end-labelled oligonucleotide, SPE5-ext (Fig. 3.7B). Transcripts of *traM* were barely visible in MC4100/pOX38-Tc at time points beyond three hours, in accordance with previous results (Frost and Manchak, 1998), and were detected as a single band of the appropriate size for *traM*, approximately 0.5 kb. However, in PD32/pOX38-Tc, *traM* mRNA is readily detectable at all time points, with many bands larger than the 0.5 kb fragment. Since these larger transcripts coincided with those seen in the *traJ* northern,

Figure 3.7 Northern analysis of transcript levels for both *traM* and *traJ*. Standing overnight cultures of MC4100/pOX38-Tc and PD32/pOX38-Tc were diluted 200-fold in fresh LB broth and incubated with shaking at 37° C. At the indicated time points, culture samples were collected and total cell RNA was isolated and analyzed by Northern blotting. The blot was probed for *traJ* (A) and *traM* (B) transcripts, as well as 16S rRNA as a loading control (C). Arrows adjacent to each blot indicate the relative position and size of RNA molecular weight standards. *traJ*-P_J indicates the position of the primary *traJ* transcript. *traM*-P_M indicates the position of the primary *traM* transcript.

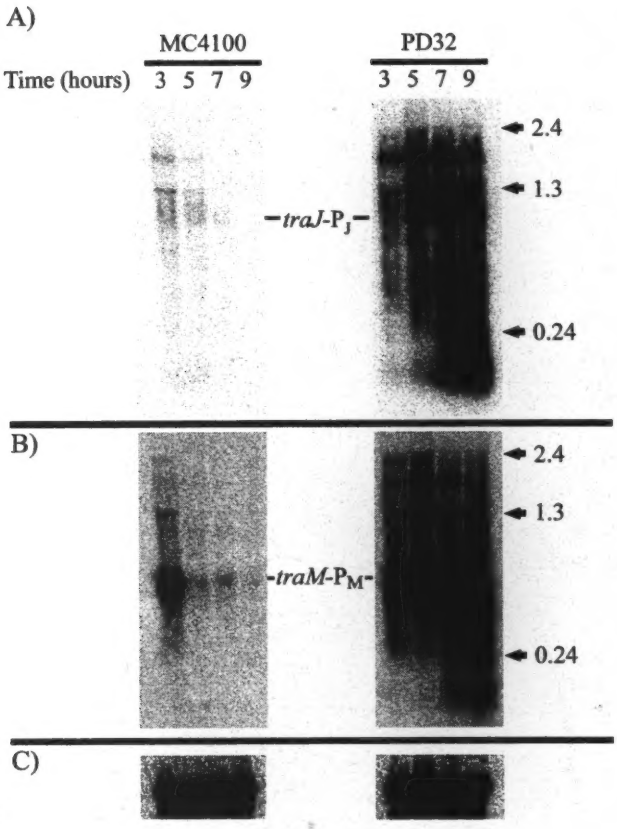
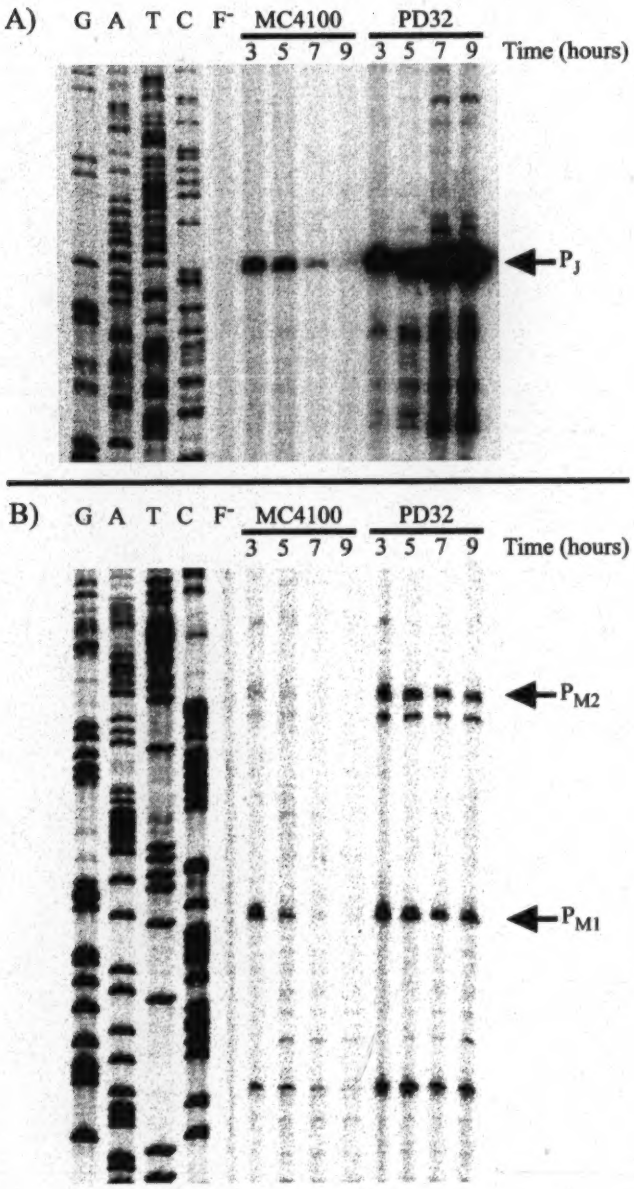

A)
MC4100
PD32
Time (hours) 3 5 7 9
3 5 7 9
2.4
1.3
traJ-P$_J$
0.24
B)
2.4
1.3
traM-P$_M$
0.24
C)

Figure 3.8 Primer extension analysis of P_M and P_J. Standing overnight cultures of MC4100, MC4100/pOX38-Tc, and PD32/pOX38-Tc were diluted 200-fold in fresh LB and incubated with shaking at 37° C. At the time points indicated above the gel, culture samples were taken and total cellular RNA was isolated and used in primer extension analysis of both the P_J (A) and P_M (B) regions. F^- refers to RNA from MC4100, without pOX38-Tc, taken at 3 hours after dilution and used as a negative control. The reaction products were then electrophoresed on a 6% polyacrylamide sequencing gel, along with sequencing reaction standards, indicated by G, A, T, and C, to the left of the primer extension reactions.


A)
G A T C F⁻
MC4100
PD32
3 5 7 9 3 5 7 9
Time (hours)
P_J
B)
G A T C F⁻
MC4100
PD32
3 5 7 9 3 5 7 9
Time (hours)
P_M2
P_M1

transcription from the *traM* promoters appears to be reading through into *traJ*, and perhaps even beyond into the *tra* operon, to *traL*, where a large transcript stability element is present (Koraimann and Hogenauer, 1989; Lee *et al.*, 1992; Frost *et al.*, 1994). This is further supported by northern data using a probe specific for *traY* transcripts, which also show these larger fragments in an *hns* mutant strain (see Chapter 4). Although the 0.5 kb fragment is detectable in MC4100/pOX38-Tc and PD32/pOX38-Tc at early time points, another, shorter transcript is detectable in PD32/pOX38-Tc throughout the growth curve. A shorter product is also detectable in primer extension assays, with a 5' terminus immediately upstream of the *traM* translational start (data not shown), but this is probably a stable degradation product, since there are no clear promoter elements in the area. Primer extension analysis confirmed that both P_{M1} and P_{M2} were derepressed in PD32/pOX38-Tc (Fig. 3.8B). Both products were undetectable in MC4100/pOX38-Tc as the culture progressed through the growth cycle, whereas each was readily detectable throughout the growth cycle in PD32/pOX38-Tc. P_{M2} appeared to be strongly affected, as it was barely detectable in wild-type cells, as was previously reported (Penfold *et al.*, 1996). However, in PD32/pOX38-Tc, the P_{M2} transcript is easily detectable, suggesting that P_{M2}, in particular, is strongly repressed by H-NS. Additional bands are likely artifacts due to stalling of the reverse transcriptase on secondary structure in the region, and not the product of additional promoters. Together, these results suggest that H-NS is a potent repressor of transcription for both *traM* and *traJ*.

3.2.7 β-galactosidase fusion analysis of *traM* and *traJ* expression

In order to better understand the reason for the discrepancies between our data and that in previous studies by Starcic-Erjavec *et al.*, (2003), a series of β-galactosidase

assays were performed using P_M and P_J fusions. Transcriptional fusions were constructed using pJLac101, a pPR9TT-based low-copy vector that avoids possible gene dosage effects (Santos *et al.*, 2001). pJLac107, a P_J fusion containing the H-NS binding sites identified by DNase I footprinting (Fig. 3.1B), was initially assayed for β-galactosidase activity. Similar to the results of Starcic-Erjavec *et al.*, (2003) in their studies on the effect of H-NS on *traJ* in pRK100, pJLac107 exhibited reduced activity in the absence of H-NS. Based on these results, it would appear that the P_J promoter in isolation is not repressed, and could be activated by H-NS, in contrast to the data above. The presence of an active *finP* promoter along with P_J in pJLac106 appears to limit the apparent activation by H-NS, but the expected repression is still not observed. The plasmid, pJLac122, a P_J-*lacZ* fusion carrying the upstream *traM* promoters, P_{M1} and P_{M2}, grew slowly, but also did not exhibit H-NS-mediated repression. Rather, as with pJLac107, pJLac122 displayed decreased activity in PD32, suggesting that H-NS acts as an activator. The presence of pOX38-Tc had no effect on these results (data not shown). However, the slow-growing phenotype observed in *hns* strains containing pJLac122 was further exacerbated by the presence of pOX38-Tc, and is thought to be due to increased levels of TraM, which are toxic to the cell (Lu and Frost, unpublished results). pJLac104, a P_{M1}-P_{M2}-*lacZ* fusion, was unaffected by the *hns* mutation. Only when P_{M1}-P_{M2} and P_J, along with *traM* and *finP* on a single fragment, were fused to *lacZ* to give pJLac110 (Fig. 3.1B), was H-NS-mediated repression apparent, with β-galactosidase activity in MC4100 at levels approximately 50% of that seen in PD32. These results suggest that a number of elements are necessary for H-NS-mediated repression of *traM* and *traJ*, including *in cis* effects between the promoter regions of *traM*, *traJ*, and *finP*.

3.3 Discussion

The results presented here suggest that the host factor, H-NS, is a potent, growth-phase-dependent regulator of F plasmid transfer gene expression in *E. coli*. The expression of the transfer genes, *traM* and *traJ*, appears to be drastically derepressed in *hns* mutant host strains, particularly as the host culture enters stationary phase. Given that TraJ is the primary plasmid-encoded activator of *tra* operon expression (Frost *et al.*, 1994), this has significant implications for the F plasmid transfer system as a whole. It suggests that H-NS sensitizes the F plasmid to the physiological state of its host, thereby preventing unwanted transfer gene expression during times of nutritional stress, such as that encountered in stationary phase. Furthermore, since an extended DNA sequence is required for H-NS-mediated repression, it seems possible that P_M and P_J are undergoing regional gene silencing.

The role of H-NS as a regulator of physiologically and environmentally dependent gene expression is well documented (Atlung and Ingmer, 1997; Hommais *et al.*, 2001). H-NS has been implicated in osmoregulation (Ueguchi and Mizuno, 1993; Lucht *et al.*, 1994), acid resistance, and RpoS stability and regulation (Barth *et al.*, 1995; Yamashino *et al.*, 1995), as well as in the control of other starvation-related genes (Hommais, *et al.*, 2001). Perhaps even more pertinent to F plasmid biology, is the role of H-NS as a repressor of plasmid-borne virulence genes in pathogenic *E. coli* and related species such as *Shigella flexneri* (Tobe *et al.*, 1993; Colonna *et al.*, 1995; Beloin and Dorman, 2003). As with F plasmid transfer, virulence gene expression is responsive to a number of environmental and physiological cues, including growth phase and temperature (Galan and Sansonetti, 1996). In the case of *S. flexneri* virulence, H-NS is known to directly

repress the essential virulence regulators, VirF and VirB (Hromockyj *et al.*, 1992; Porter and Dorman, 1994). VirF is a transcriptional activator which upregulates the expression of *virB* (Adler *et al.*, 1989; Tobe *et al.*, 1993). VirB, in turn, activates structural virulence genes, which have recently been shown to also be repressed by H-NS (Beloin and Dorman, 2003). Although the *vir* and *tra* regulatory systems themselves do not display significant homology, the similar cues to which they respond suggest regulation by similar chromosomally-encoded mechanisms.

Immunoblot analysis shows a drastic increase in cellular TraJ levels throughout the growth cycle and at 30°C in *hns* mutant donor cells. TraM levels are also increased in the mutant strain, but not to the degree that TraJ levels are. There is not a direct correlation between the constitutive TraJ levels and mating ability, in that mating ability does eventually decrease, even in an *hns* mutant donor, however this is thought to be due, at least in part, to a growth phase-dependent modification of TraJ, as isoelectric focusing analysis suggests there are multiple forms of TraJ appearing at different points in the growth cycle (see Chapter 9). This modification event would help explain, at least in part, why transfer ability has declined long before there is a significant change in TraJ levels. Northern blot and primer extension analyses of *traM* and *traJ* mRNA suggest that transcription of both genes is derepressed throughout the growth curve in *hns* mutant strains, whereas transcription in wild-type cells occurs only briefly in early exponential phase. Furthermore, a number of larger transcripts were detected in the *hns* mutant, which suggests that the *traM* promoters drive read-through transcription into *traJ* and beyond, as suggested previously in the F-like plasmid, R100 (Stockwell *et al.*, 2000). H-NS appears to limit this read-through, isolating the transcription of *traM* and *traJ*. H-NS

also represses P_{M2} more strongly than P_{M1}, as it appears to be relatively inactive in wild-type cells, but is strongly derepressed in the *hns* mutant. This might allow for high levels of *traM* transcription when cellular conditions are inappropriate for H-NS binding. The finding that TraJ levels were derepressed in the *hns* mutant when grown at 30°C suggests that H-NS regulates *traJ* in a temperature-dependent manner. This may be due to temperature-dependent fluctuations in curvature, as has been suggested for H-NS-mediated thermoregulation of *virF* in *Shigella flexneri*.

Sequence analysis and binding studies show that H-NS binds upstream of *traM* at sites that overlap IHF binding sites, suggesting a possible mode of action (Fig. 3.3A). Given that IHF and H-NS antagonize each other in the control of *virF* and *virB* in *S. flexneri* (Porter and Dorman, 1997), a similar mode of action might exist in F. IHF is essential for plasmid nicking and transfer (Howard *et al.*, 1995; Nelson *et al.*, 1995). Furthermore, IHF has been shown to positively influence transcription of *traJ* and *traM* in the F-like plasmid, R100 (Dempsey and Fee, 1990). IHF is also thought to positively influence *tra* operon expression since piliation and *traY-lacZ* fusion activity have both been observed to decrease in *himA/hip* mutants, although this may be explained in part by possible changes in TraJ levels in such mutants (Gamas *et al.*, 1987; Dempsey and Fee, 1990; Silverman *et al.*, 1991a).

EMSA and DNase I footprinting analysis indicate that H-NS binds extensively to a 150 bp region at the *traJ* promoter, P_J (Fig. 3.3B). The binding sites identified by DNase I footprinting analysis overlap the predicted binding sites for the *traJ* transcriptional activators, Lrp and CRP (Camacho and Casadesus, 2002; Starcic *et al.*, 2003; Starcic-Erjavec *et al.*, 2003). This suggests that H-NS may act, at least in part, by

competing with Lrp and CRP to bind the P_J region. However, based on transcriptional fusion analysis, this region alone is not sufficient for H-NS-mediated repression as it undergoes H-NS-mediated activation when fused to *lacZ*, which is in agreement with the findings of Starcic-Erjavec *et al.*, (2003). Still, all other data, including immunoblot and northern analysis, as well as mating assays, suggest that H-NS acts to repress *traJ* transcription. It would appear that the transcriptional fusion data of Starcic-Erjavec *et al.*, (2003) are the result of examining P_J out of the context of the F regulatory loop. Whereas P_M and P_J alone do not undergo H-NS-mediated repression, constructs with the two promoters *in cis* to one another, along with a functional *finP* gene, exhibit H-NS-mediated repression. Based on these results, we suggest that H-NS might act as a silencer, as described by Rimsky *et al.*, (2001), which binds to a number of nucleation sites, such as the predicted curves. Once the amount of bound H-NS reaches a certain threshold, H-NS will begin to polymerize outward along the DNA from its early binding sites and repress local promoters, including P_J, and P_M. Given the *lacZ* fusion results, P_J and P_M alone may not be able to bind sufficient H-NS to reach the required threshold to promote nucleation. Additional nucleation sites may be necessary for H-NS-mediated repression. Alternatively, the apparent requirement for an active form of *finP* to be present for H-NS-mediated repression to occur may reflect some sort of structural change in local DNA due to altered supercoiling since P_{finP} fires into both P_J and P_M, through *finP*, which contains extensive secondary structure. P_{finP} also appears to attenuate TraM toxicity, as observed in pJLac122, either by extended antisense transcripts, or by interference at the transcriptional level, such as during RNA polymerase transcription of

finP, moving towards P_M and generating positive superhelicity, which would inhibit RNA polymerase binding and transcriptional initiation at those promoters.

H-NS affinity for P_J and P_M may fluctuate in response to other proteins binding to these promoters or to changes in DNA supercoiling, allowing H-NS bound at those sites to switch from an activation complex to a repressor complex. DNA supercoiling is known to respond to cellular free energy, and fluctuations in superhelicity would likely influence curvature, and hence H-NS binding (van Workum *et al.*, 1996). As cells progress through the growth cycle, the affinity of H-NS for P_M and P_J may increase, either in response to a decrease in bound competitor proteins, or in response to altered curvature due to decreased supercoiling. In accordance with this model, transcriptional activators in the F plasmid *tra* regulatory circuit, such as TraY, and the host factors Lrp and CRP, could be acting as antagonists to H-NS binding and silencing during exponential growth. Thus, these activators may not activate transcription via RNA polymerase, but instead act as derepressors by competing with H-NS for access to the P_M and P_J promoter regions.

It is possible that part of the H-NS-mediated repressive effect is due to indirect effects. The gene encoding Lrp, a *traJ* activator in the F-like plasmids, pSLT and pRK100 (Camacho and Casadesus, 2002; Starcic-Erjavec *et al.*, 2003), has been shown to be part of the H-NS regulon (Hommais *et al.*, 2002). Increased Lrp levels in *hns* mutant strains might be responsible for part of the increase in TraJ. However, this does not explain the apparent contextual dependence of H-NS mediated repression exhibited in the transcriptional fusion data.

Data from plasmids like pRK100 are further complicated by the presence of Hha and Hha/YmoA family proteins. Hha has been shown to interact directly with H-NS, and is thought to modulate its activity (Nieto *et al.*, 2002). Hha homologs have been identified in numerous conjugative plasmids, including the F-like pRK100 and R100, where the Hha homolog, RmoA, has been shown to positively regulate R100 plasmid transfer (Nieto *et al.*, 1998). Although no Hha homolog has been identified in the F plasmid itself, these proteins may act to modulate or limit H-NS-mediated repression in other F-like plasmids. These results serve to demonstrate that studying a complex regulatory loop such as that in F must be done in the context of the entire plasmid. Still, it is clear from the data presented that H-NS acts as a repressor of *tra* gene expression, and that it plays a role in converting F^+ cells to "F^- phenocopies" in stationary phase.

Chapter 4: Characterization of the opposing roles of H-NS, TraJ, and TraY in the transcriptional regulation of F plasmid *tra* gene expression*

* Portions of this chapter were published: Will, W.R., and Frost, L.S., (2006) *J Bacteriol.* **188:** 507-514.

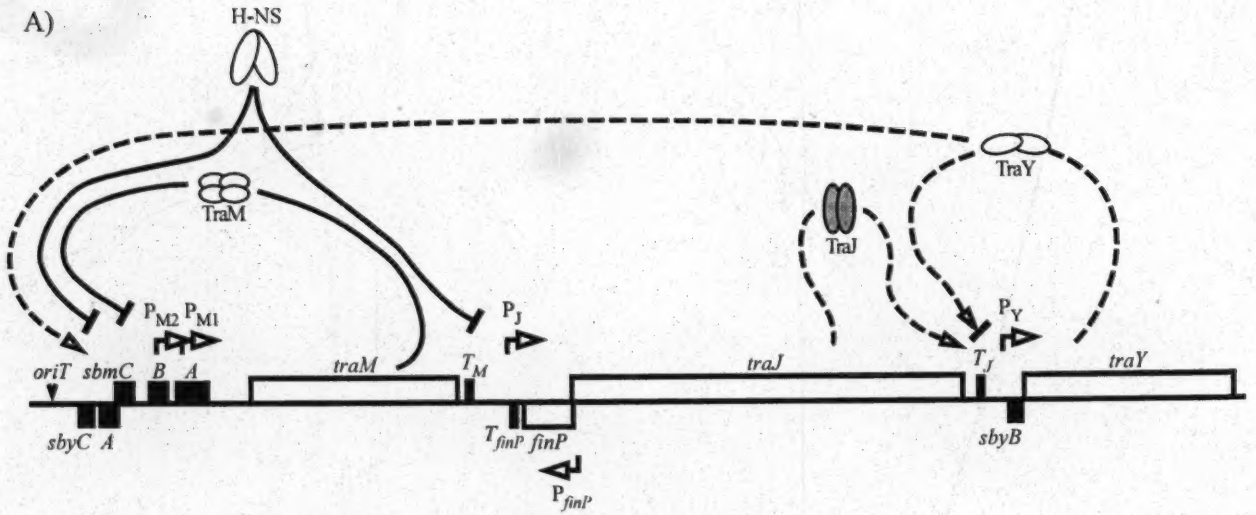
4.1 Introduction

Bacterial conjugation allows the transfer of DNA from a donor to a recipient cell by way of a complex conjugative apparatus composed of an F-type IV secretion system and auxiliary proteins for DNA metabolism and regulation (Lawley *et al.*, 2003). In the case of the conjugative F plasmid of *Escherichia coli*, the expression of genes encoding components of this apparatus is extremely growth phase-dependent, dropping to undetectable levels as donor cell cultures enter stationary phase (Frost and Manchak, 1998; Will *et al.*, 2004). This was recently shown to be due, at least in part, to growth-phase dependent transcriptional silencing by the host nucleoid-associated protein, H-NS (Will *et al.*, 2004). H-NS is uniquely suited to regulating transcription in a wide array of mobile genetic elements, since it shows a preference for intrinsically curved, AT-rich DNA, which is a common feature of many promoters (Pon *et al.*, 1988; Owen-Hughes *et al.*, 1992; Dorman, 2004). H-NS binds preferentially to these curved sequences and nucleates outwards, silencing local promoters (Williams and Rimsky, 1997; Badaut *et al.*, 2002). Whereas cellular H-NS levels are relatively static throughout growth, at approximately 20,000 molecules per cell, the regions of curvature to which H-NS binds are dynamic, responding to both environmental and nutritional cues via fluctuations in chromosomal supercoiling and protein binding (Free and Dorman, 1995; Williams and Rimsky, 1997). This property allows it to repress many genes, including those acquired via mobile elements in a dynamic, physiologically responsive manner. As a result, the cell may be capable of avoiding non-essential gene expression when under environmentally or nutritionally limiting conditions.

F plasmid transfer gene expression is regulated by three plasmid-encoded regulators: TraJ, TraY, and TraM (Fig. 4.1A) (Frost *et al.*, 1994). TraJ is the primary activator, expressed from its own monocistronic operon. It is required for transcription of the 33 kb transfer (*tra*) operon, which encodes all of the components of the transfer apparatus, from the P_Y promoter, (Willetts, 1977; Silverman *et al.*, 1991a). In most F-like plasmids, *traJ* is subject to post-transcriptional regulation via the FinOP antisense RNA system (van Biesen and Frost, 1994). However, the gene encoding the RNA chaperone, *finO*, is disrupted in the F plasmid by the insertion of an IS*3* element, which results in the subsequent derepression of TraJ synthesis (Cheah and Skurray, 1986). The first gene in the *tra* operon, *traY*, encodes the secondary regulator of P_Y, TraY. Early results suggested that TraY activates *tra* operon expression, although more recent studies suggest that TraY can also act as a repressor (Silverman and Sholl, 1996; Taki *et al.*, 1998). TraY also activates expression of *traM*, a monocistronic operon upstream of *traJ*, which encodes the autorepressor TraM (Penfold *et al.*, 1996). In addition to its regulatory role, TraY is essential for mating since it is a critical component of the relaxosome, a nucleosomal complex that forms at the plasmid origin of transfer and is responsible for nicking and unwinding the plasmid DNA in preparation for transfer (Howard *et al.*, 1995; Nelson *et al.*, 1995). TraM is essential for transfer and is part of the mature relaxosome (Fekete and Frost, 2002). It binds the coupling protein, TraD, thereby linking the relaxosome to the transferosome, a protein complex that spans the cell envelope and forms the channel for DNA transport during conjugation (Disque-Kochem and Dreiseikelmann, 1997; Lu and Frost, 2005).

Figure 4.1 The F plasmid regulatory circuit (A). A positive effect on transcription is indicated by dashed lines ending in arrowheads, whereas a negative effect is indicated by solid lines ending in bars. Conflicting evidence suggests that TraY can act as both an activator and a repressor at the *tra* operon promoter, P_Y, and this is indicated by a dashed line ending in both an arrowhead and a bar. The *traY*, *traJ*, and *finP* promoters are indicated by P_Y, P_J, P_{finP}, respectively. Transcription of *traM* is driven by two promoters, indicated by P_{M1} and P_{M2}. The *traM*, *traJ*, and *finP* transcriptional terminators are indicated by T_M, T_J, and T_{finP}, respectively. TraM binding sites are labeled as *sbmA*, *-B*, and *-C*, whereas TraY binding sites are labeled as *sbyA*, *-B*, and *-C*. Other regulatory factors not directly relevant to this study have been omitted. The effect of H-NS on *tra* operon promoter activity was examined using *lacZ* transcriptional fusion constructs (B). The coordinates of the promoter fragment termini relative to the transcriptional start site at P_Y (+1) are indicated. The size and position of each promoter fragment relative to the genetic map above is indicated by solid lines. Standing overnight cultures of MC4100 and PD32 containing each construct were diluted into fresh LB broth and incubated with shaking at 37°C. Samples were taken at 3 and 7 hours of growth and β-galactosidase activity assays were then performed. The results are given in Miller Units (MU) (Miller, 1972), and indicate the average and standard deviation of three separate experiments.

A)



B)

Coordinates	3 hours		7 hours		
	MC4100	PD32	MC4100	PD32	
-394/+504	22 +/- 1	459 +/- 56	51 +/- 6	1356 +/-47	pRWPY101
-77/+504	<1	<1	<1	<1	pRWPY102
-394/+205	275 +/-14	2926 +/- 344	336 +/- 13	5478 +/- 560	pRWPY103

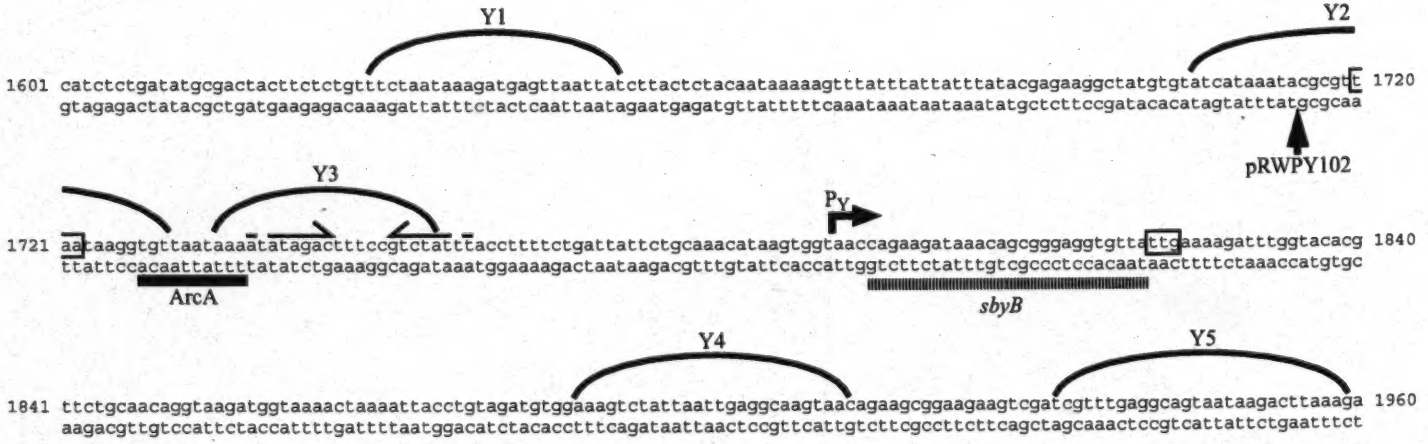
The mechanism for controlling transcription from P_Y, the *tra* operon promoter, remains undefined. Earlier studies hypothesized that a nucleosomal complex forms upstream of P_Y, altering local supercoiling and repressing transcription (Gaudin and Silverman, 1993). While the exact mechanism of TraJ activity has not been elucidated, it has been suggested that TraJ opposes the formation of this complex to allow the initiation of transcription from P_Y (Gaudin and Silverman, 1993). In this study, we present data suggesting that H-NS forms the repressor complex previously hypothesized, repressing the *tra* operon promoter, P_Y, in a growth phase-dependent manner, as previously observed for the F plasmid promoters, P_M and P_J, responsible for driving transcription of *traM* and *traJ*, respectively (Will *et al.*, 2004). Furthermore, we present evidence suggesting that the *tra* activator, TraJ, serves, at least in part, to counter the repressive effects of H-NS at the P_Y promoter.

4.2 Results

4.2.1 H-NS binds to intrinsically curved DNA at P_Y

Since H-NS binds preferentially to regions of intrinsic curvature (Owen-Hughes *et al.*, 1992), the P_Y region was examined for predicted intrinsic curves using the BEND-IT curvature prediction program (http://www.icgeb.org/dna/bend_it.html). BEND-IT identified approximately 5 regions with curvature of approximately 6° or greater per helical turn near P_Y (Fig. 4.2). The bend center of Y1 is located at position -152 relative to P_Y, upstream of the *traM* stop codon. The bend center of Y2, located at position -74, overlaps the *traJ* stop codon and Y3 is immediately downstream, centered at position -67. Y2 and Y3 also overlap the binding site for the positive activator, ArcA (Lynch and Lin, 1996; Strohmaier *et al.*, 1998), as well as an inverted repeat which has been identified as

Figure 4.2 The P_Y region contains significant intrinsic curvature. The BEND-IT computer program (http://www.icgeb.org/dna/bend_it.html) was used to identify sequences of DNA with significant intrinsic curvature (greater than 5° per helical turn). Five significant bends were identified in the region examined, designated Y1 through 5. The bends are indicated by solid arced lines immediately above the sequence. A binding site for the host protein, ArcA, identified by previous studies (Lynch and Lin, 1996; Strohmaier *et al.*, 1998), is indicated by a solid bar immediately beneath the sequence, while the TraY binding site, *sbyB*, is indicated by a dashed line. The *traJ* stop and *traY* start codons are enclosed in boxes. The upstream terminus of the promoter fragment in pRWPY102 is indicated by a vertical arrow below the sequence. The sequence is numbered relative to the position of a BglII site at the start of the transfer region and all other features are indicated as previously described (Frost *et al.*, 1994).


Y1
Y2
1601 catctctgatatgcgactacttctctgtttctaataaagatgagttaattatcttactctacaataaaaagtttatttattatttatacgagaaggctatgtgtatcataaatacgcgtt 1720
gtagagactatacgctgatgaagagacaaagattatttctactcaattaatagaatgagatgttatttttcaaataaataataaatatgctcttccgatacacatagtatttatgcgcaa
pRWPY102
Y3
P_Y
1721 aattaaggtgttaataaaatatagactttccgtctatttaccttttctgattattctgcaaacataagtggtaaccagaagataaacagcgggaggtgttattgaaaagatttggtacacg 1840
ttattccacaattattttatatctgaaaggcagataaatggaaaagactaataagacgtttgtattcaccattggtcttctatttgtcgccctccacaataacttttctaaaccatgtgc
ArcA
sbyB
Y4
Y5
1841 ttctgcaacaggtaagatggtaaaactaaaattacctgtagatgtggaaagtctattaattgaggcaagtaacagaagcggaagaagtcgatcgtttgaggcagtaataagacttaaaga 1960
aagacgttgtccattctaccattttgattttaatggacatctacacctttcagataattaactccgttcattgtcttcgccttcttcagctagcaaactccgtcattattctgaatttct

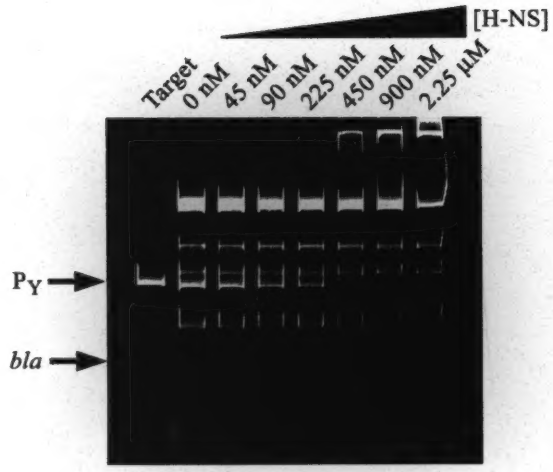
a binding site for TraJ in the F-like plasmid, R100 (Taki *et al.*, 1998). The final two regions of significant curvature, Y4 and Y5, are centered at +108 and +154, respectively, within the *traY* gene.

To determine whether or not H-NS preferentially binds to this region, a competitive electrophoretic mobility shift assay was performed. Equimolar amounts of a PCR-amplified fragment containing the P_Y target region with the five predicted bends and competitor DNA were mixed, incubated with increasing concentrations of H-NS, and separated on a polyacrylamide gel (Fig. 4.3). pBR322 digested with TaqI and SspI was used as a competitor and a positive control, as one of the digested fragments contains the *bla* promoter, which is known to display significant curvature and be bound preferentially by H-NS (Zuber *et al.*, 1994). H-NS bound the P_Y target at lower concentrations than the *bla* positive control fragment, suggesting that H-NS specifically binds this region.

4.2.2 H-NS down-regulates transcription from P_Y

Although previous studies have shown that TraY levels are increased in *hns* donor cultures as they enter stationary phase, this might reflect secondary effects due to an increase in cellular levels of the *tra* activator TraJ (Will *et al.*, 2004). However, since H-NS preferentially bound the P_Y region, H-NS might also directly repress transcription of the *tra* operon from P_Y. The effect of H-NS on *tra* operon transcription was determined by isolating total cellular RNA at regular intervals throughout the growth curve from wild-type (MC4100) and *hns* (PD32) strains containing pOX38-Tc, a derivative of the F plasmid. The RNA was then used in northern blot analysis with the end-labeled oligonucleotide, RWI78, a *traY*-specific probe. Transcript levels in wild-type donor cells decreased rapidly as the cells progressed through the growth cycle, reaching undetectable

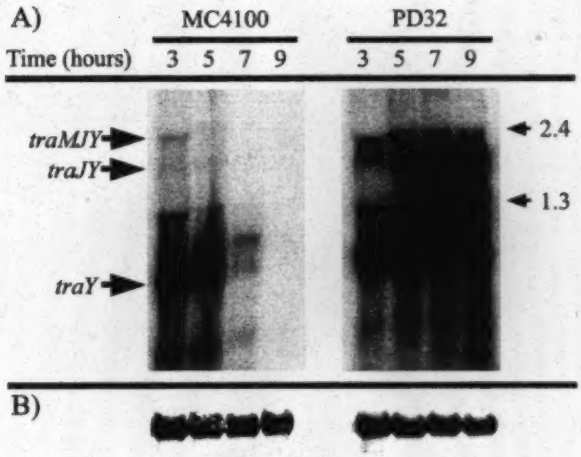
Figure 4.3 H-NS binds preferentially to the P_Y region. Competitive electrophoretic mobility shift assays were performed by incubating equimolar amounts of P_Y target DNA and TaqI-SspI-digested pBR322 in increasing concentrations of H-NS, indicated above the gel, for 20 minutes at room temperature. The resulting complexes were then resolved by electrophoresis in a 7.5% TBE-polyacrylamide gel. The 389 bp P_Y target fragment is indicated, as is the 215 bp *bla* competitor fragment, to which H-NS binds specifically.


[H-NS]
Target
0 nM
45 nM
90 nM
225 nM
450 nM
900 nM
2.25 μM
P_Y
bla

levels after 9 hours (Fig. 4.4). In the *hns* mutant strain, levels of the primary transcript were present for a prolonged period, and were still detectable after 9 hours of growth. The small size of the *tra* operon transcript (~ 1.0 kb) that begins with *traY* is due to the rapid processing of *tra* mRNA at a site located within *traL* (Koraimann and Hogenauer, 1989). Several larger transcripts also appeared to accumulate in the *hns* donor cells during stationary phase. These larger species were also detected with *traJ* and *traM*-specific probes and were therefore thought to be read-through products from the upstream P_J and P_M promoters (Will *et al.*, 2004). H-NS appears to limit read-through by separating the *traM*, *traJ* and the *tra* operons into distinct transcriptional domains by binding and sequestering their respective promoter regions.

To address whether H-NS acts directly upon P_Y or acts through *traJ*, a series of P_Y-*lacZ* transcriptional fusions were constructed. Activity was assayed for both minimal and extended promoter regions, with and without a complete *traY* ORF (Fig. 4.1B). pRWPY101 contains an extended promoter region that includes the 3' half of the *traJ* gene, all of the predicted curves, Y1 to Y5, and a functional *traY* gene to supply TraY. pRWPY102 contains a minimal promoter region, which lacks almost all of *traJ,* Y1 and part of Y2, but contains a complete *traY* gene. pRWPY103 contains the extended promoter region of pRWPY101, but lacks a complete *traY* gene. A construct containing a minimal promoter without a functional *traY* gene was unstable, suggesting that the absence of either TraY protein or sequences within *traY* destabilized this construct. The β-galactosidase activity of each fusion was assayed in MC4100 and PD32 cells in exponential phase after three hours of growth, and in early stationary phase, after seven hours of growth. The test strains did not contain the F plasmid, to prevent possible

Figure 4.4 H-NS represses *traY* transcription as donor cells approach stationary phase. Standing overnight cultures of both MC4100 and PD32 containing pOX38-Tc were diluted into fresh LB and incubated at 37°C with shaking. At the indicated time-points, culture samples were removed, from which total cellular RNA was purified. This RNA was used in a northern blot that was probed for *traY* containing transcripts (A). The relative positions of a molecular weight marker are indicated on the right of the blot. *traMJY* indicates a transcript which is detected by *traM*, *traJ*, and *traY*-specific probes, and is predicted to be a transcriptional read-through product originating from P_M (data not shown). *traJY* indicates a transcript which hybridizes with both *traY* and *traJ*-specific probes (data not shown), and is predicted to be a transcriptional read-through product originating from P_J. *traY* indicates a transcript which is only detected by a *traY*-specific probe, and is predicted to be the mature, processed *traY* transcript originating from P_Y. 23S rRNA was visualized on membranes prior to hybridization by staining with Blot Stain Blue (Sigma-Aldrich) to check for loading and transfer quality (B).


A)
MC4100
PD32
Time (hours)
3 5 7 9
3 5 7 9
traMJY
traJY
traY
2.4
1.3
B)

secondary effects from altered TraJ levels in PD32. Mutation of *hns* resulted in derepression of two of the three constructs, pRWPY101 and pRWPY103 (Fig. 4.1B). pRWPY102 exhibited no detectable activity in either strain. Whereas TraY is generally thought to be an activator of *tra* operon expression, (Silverman and Sholl, 1996), these transcriptional fusion results suggested an additional role as an autorepressor. The inclusion of *traY* in pRWPY101 resulted in a decrease in activity of approximately 5-10 fold compared to pRWPY103, depending on the host strain and time the sample was taken. The decrease is not due solely to the inclusion of additional H-NS binding sites, which would repress β-galactosidase activity, since the decrease is also observed in PD32.

4.2.3 TraJ is unnecessary for F plasmid transfer from an *hns* host

In many H-NS-regulated systems, H-NS is opposed by one or more positive regulators, which act to compete with and block H-NS from binding, an example being the antagonism of H-NS by Fis at *rrnB* P1 (Afflerbach *et al.*, 1998; Schroder and Wagner, 2000). We considered the possibility that TraJ opposed H-NS-mediated repression, and that TraJ, considered essential for F plasmid transfer (Willetts, 1977; Gaffney *et al.*, 1983; Silverman *et al.*, 1991a), might be unnecessary in an *hns* mutant host. To test this hypothesis, we examined the mating efficiency of an F plasmid variant containing an amber mutation in *traJ*, F*lac traJ90*, in both MC4100 and the *hns* mutant strain PD32 during exponential phase. F*lac traJ90* is a well-characterized F plasmid mutant that maintains the sequence context and spacing of wild-type F*lac*, and has been shown in previous studies to be incapable of transfer, but is fully complementable when TraJ is supplied *in trans* (Achtman *et al.*, 1971). Although transfer was barely detectable

in MC4100, it was partially restored in PD32 by approximately 4 orders of magnitude (Table 4.1). This suggests that TraJ could serve, at least in part, to oppose H-NS at P_Y. However, mating efficiency was approximately 20 to 50-fold lower than for wild-type plasmids. This suggests that either TraJ has a role as an activator in the absence of *hns*, or that the *hns* mutation has other secondary effects on the F plasmid. These results also indicate that H-NS acts directly at P_Y. If the H-NS-dependent changes in *traY* transcript levels were due solely to secondary effects of H-NS on TraJ, transfer of F*lac traJ90* should be similar in both host strains.

4.2.4 TraJ opposes H-NS in the regulation of *tra* gene expression

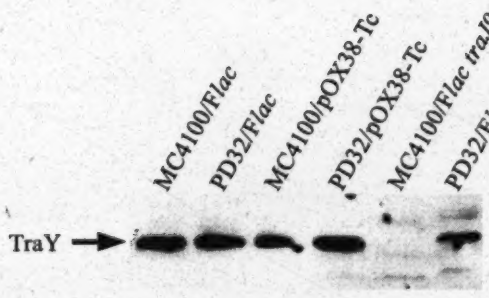
To establish that TraJ opposes H-NS at the level of *traY* expression, TraY levels were assayed via immunoblot analysis in samples collected from exponential phase cultures of MC4100 and PD32 containing F*lac traJ90* (Fig. 4.5A). TraY was undetectable in MC4100/F*lac traJ90* cultures but was restored to nearly normal levels in PD32/F*lac traJ90* cells. Similarly, TraM levels, which require TraY for maximal expression (Penfold *et al.*, 1996), were significantly reduced in MC4100 cultures containing F*lac traJ90*, but were restored in PD32 hosts (Fig. 4.5B). To confirm these results at the transcriptional level, total cellular RNA was extracted from exponential phase MC4100/F*lac traJ90* and PD32/F*lac traJ90* cultures and examined via Northern blot analysis. Probing the membrane with [^{32}P]-end-labeled RWI78, the *traY*-specific probe, revealed that *traY* was undetectable in MC4100/F*lac traJ90*, whereas *traY* was present in PD32/F*lac traJ90* (Fig. 4.6A). These results indicate that TraJ opposes H-NS-mediated repression of *tra* operon transcription from P_Y.

Table 4.1 Plasmid transfer of F*lac traJ90* is partially restored in an *hns* host strain.

Donor strain	Mating efficiency (transconjugants/donor)
MC4100/pOX38-Tc	0.21
PD32/pOX38-Tc	0.16
MC4100/F*lac*	0.43
PD32/F*lac*	0.41
MC4100/F*lac traJ90*	4.0×10^{-7}
PD32/F*lac traJ90*	8.7×10^{-3}

Figure 4.5 Immunoblot analysis of TraM and TraY from mutant plasmids in wild-type and *hns* mutant donor cells. Standing overnight cultures of MC4100 and PD32 containing F*lac traJ*90 and pOX38*traY244*, as well as the wild-type plasmids F*lac* and pOX38-Tc, were diluted into fresh LB broth and grown to exponential phase. Samples equivalent to 0.1 OD_{600} were then collected, pelleted, and probed for TraY (A) or TraM (B) protein via immunoblot analysis.

A)


MC4100/F'lac
PD32/F'lac
MC4100/pOX38-Tc
PD32/pOX38-Tc
MC4100/F'lac traJ90
PD32/F'lac traJ90
TraY

B)

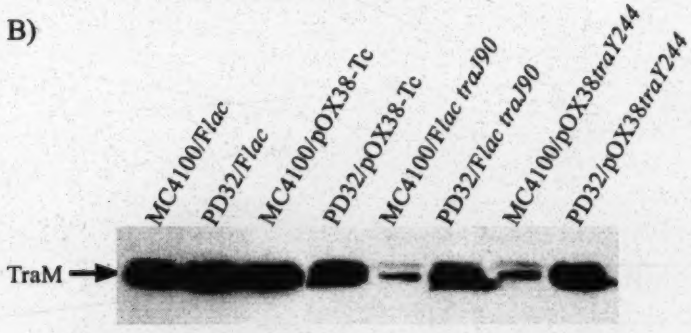
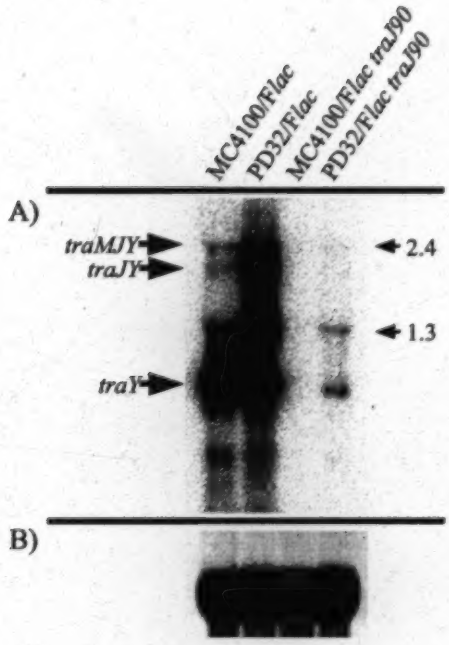

MC4100/F'lac
PD32/F'lac
MC4100/pOX38-Tc
PD32/pOX38-Tc
MC4100/F'lac traJ90
PD32/F'lac traJ90
MC4100/pOX38traY244
PD32/pOX38traY244
TraM

Figure 4.6 TraJ opposes H-NS-mediated repression of *tra* gene expression. Total RNA was extracted from exponential phase cultures of the wild-type host, MC4100, as well as the *hns* mutant strain, PD32, carrying either the wild-type plasmid, F*lac*, or the *traJ* mutant plasmid, F*lac traJ*90, and analyzed via northern blot. Membranes were probed for *traY* (A), and stained for 23S rRNA (B) as a loading control. The relative positions of a molecular weight marker are indicated to the right of the blot. *traMJY* indicates a transcript which is detected by *traM*, *traJ*, and *traY*-specific probes, and is thought to be a transcriptional read-through product from P_M. *traJY* indicates a transcript which is detected by both *traY* and *traJ*-specific probes, and is thought to be a transcriptional read-through product originating from P_J. *traY* indicates a transcript which is only detected by a *traY*-specific probe, and is predicted to be the mature, processed *traY* transcript originating from P_Y.


MC4100/Flac
PD32/Flac
MC4100/Flac traJ90
PD32/Flac traJ90
A)
traMJY
traJY
traY
2.4
1.3
B)

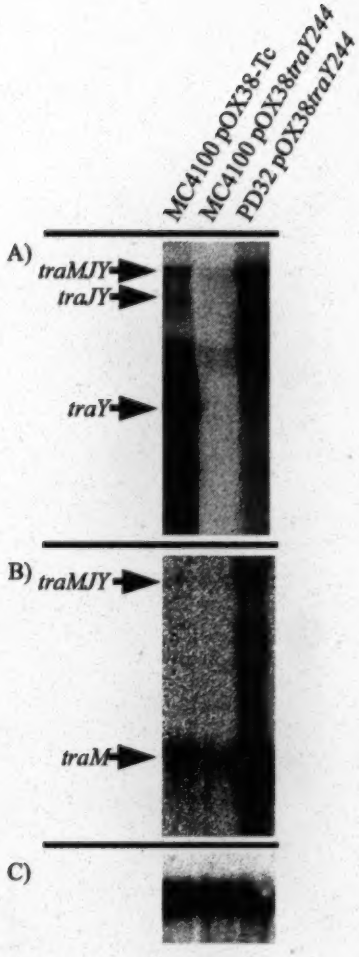
4.2.5 TraY opposes H-NS-mediated repression of *tra* gene expression

To determine if TraY acts to oppose H-NS-mediated repression at either P_Y or P_M in a manner similar to TraJ, a variant of pOX38 containing a disrupted *traY* gene, pOX38*traY244*, was examined in both MC4100 and PD32. Northern blot analysis of exponential phase cultures using both *traY*-specific (Fig. 4.7A) and *traM*-specific (Fig. 4.7B) probes demonstrated that whereas transcript levels in MC4100/pOX38*traY244* were decreased relative to the wild-type plasmid, pOX38-Tc, they were at least partially restored in PD32/pOX38*traY244*. In the case of *traM* expression, transcript levels were actually higher in PD32/pOX38*traY244* than in the wild-type MC4100/pOX38-Tc. It should be noted that the altered *traY* band pattern is due to the insertion of a *kan* cassette, disrupting the gene. As a result, the exact transcript identities cannot be confirmed for pOX38*traY244*. In an attempt to confirm these findings, immunoblot analysis was also performed with MC4100 and PD32 containing pOX38*traY244*. TraM levels, which were decreased in MC4100/pOX38*traY244*, were clearly restored, suggesting that TraY opposes H-NS-mediated repression of P_M (Fig. 4.5B). Unfortunately, immunoblotting for TraY was not possible due to the disruption of *traY*. Similarly, mating efficiency was not assayed as TraY is an essential component of the relaxosome (Howard *et al.*, 1995; Nelson *et al.*, 1995).

4.3 Discussion

In addition to its previously described effects on the *traM* and *traJ* promoters, H-NS also appears to repress F plasmid transfer gene expression at the *tra* operon promoter, P_Y. Furthermore, the findings presented here suggest a specific role for TraJ, the primary activator, in opposing H-NS-mediated repression of P_Y. Whereas TraJ was previously

Figure 4.7 TraY opposes H-NS-mediated repression of *tra* gene expression. Total RNA was extracted from exponential phase cultures of the wild-type host, MC4100, as well as the *hns* mutant strain, PD32, carrying either the wild-type plasmid, pOX38-Tc, or the *traJ* mutant plasmid, pOX38*traY244*, and analyzed via northern blot. Membranes were probed for *traY* (A), *traM* (B), and stained for 23S rRNA (C) as a loading control. The relative positions of a molecular weight marker are indicated to the right of the blot. *traMJY* indicates a transcript which is detected by *traM*, *traJ*, and *traY*-specific probes, and is thought to be a transcriptional read-through product from P_M. *traJY* indicates a transcript which is detected by both *traY* and *traJ*-specific probes, and is thought to be a transcriptional read-through product originating from P_J. *traY* indicates a transcript which is only detected by a *traY*-specific probe, and is predicted to be the mature, processed *traY* transcript originating from P_Y. It should be noted that *traY* in pOX38*traY244* contains a *kan* cassette, resulting in altered transcript sizes. *traM* indicates a transcript which is only detected by a *traM* specific probe.


MC4100 pOX38-Tc
MC4100 pOX38traY244
PD32 pOX38traY244
A)
traMJY
traJY
traY
B)
traMJY
traM
C)

thought to be essential for transfer gene expression and plasmid transfer (Willetts, 1977), it is not required for transfer gene expression in an *hns* mutant. A mechanism for TraJ activity has not been established; however, these results are in keeping with models presented for TraJ function. Gaudin and Silverman (1993) first suggested that TraJ served to oppose the formation of an undefined nucleosomal complex. The authors were able to correlate the positive effect of TraJ *in vivo* with a requirement for P_Y to be supercoiled to drive transcription *in vitro*. This suggested that an unknown repressor complex served to alter local DNA supercoiling. This alteration was antagonized by TraJ, thereby allowing the P_Y region to adopt a more transcriptionally active topology. Modulation of supercoiling has previously been suggested as a general mode of action for H-NS-mediated gene repression (Tupper *et al.*, 1994; Mojica and Higgins, 1997). Thus, H-NS might be involved in this nucleosomal complex. However, this does not eliminate other possible mechanisms for H-NS-mediated repression at P_Y. The arrangement of the five predicted curves located both upstream and downstream of P_Y might facilitate the trapping of RNA polymerase, as previously described for the *rrnB* P1 promoter region (Dame *et al.*, 2002). This mechanism proposes that the promoter DNA wraps around the RNA polymerase, promoting open complex formation during transcription initiation (Coulombe and Burton, 1999), and forming a DNA loop which brings H-NS bound at both the upstream and downstream sites into close proximity. This promotes strand-bridging by H-NS and traps the RNA polymerase bound at the promoter (Dame *et al.*, 2005). These two mechanisms are not mutually exclusive and both might be involved in H-NS-mediated repression of P_Y.

The sequences of known TraJ proteins of the F-like plasmids are exceptionally dissimilar, and bear little homology to other known proteins (Frost *et al.*, 1994). Although the F-like TraJ proteins share a putative helix-bend-helix DNA binding motif (Frost *et al.*, 1994), DNA binding has not been demonstrated *in vitro*, except for the F-like plasmid, R100 TraJ, at low pH (Taki *et al.*, 1998). Given that H-NS has already been shown to interact with a number of seemingly dissimilar and unlikely partners, such as the Hha/YmoA family of proteins (Nieto *et al.*, 2000; Nieto *et al.*, 2002), the RNA binding protein, Hfq (Muffler *et al.*, 1996a), and the flagellar motor protein, FliG (Donato and Kawula, 1998), TraJ might also interact with H-NS at P_Y, forming a stable DNA-protein complex only when H-NS is present. This would be similar to that suggested for the Hha family of proteins (Nieto *et al.*, 2000; Nieto *et al.*, 2002), which prevent formation of a repression complex thereby allowing transcription to be initiated.

Although the data presented here suggest that transcription from P_Y is lower in an *hns traJ* mutant strain than in an *hns* strain, this does not necessarily mean that TraJ has an activational role at P_Y in addition to opposing H-NS. Reduced *tra* operon expression may be due to StpA, an H-NS paralog, which is normally repressed by H-NS, but is overexpressed in *hns* mutant strains (Free and Dorman, 1997; Sonden and Uhlin, 1996). Whereas the mutation of *stpA* alone has little effect on F plasmid transfer or host cell growth, F^+ cells containing an *hns stpA* double mutation grow very slowly, much more so than either F^+ *hns* or F^- *hns stpA* cultures, suggesting partial repression by StpA in *hns* mutant host cells (data not shown). Although TraJ might also be capable of antagonizing increased StpA-mediated repression in *hns* mutant donors, P_Y might not be fully derepressed in an *hns traJ* mutant donor cell.

Another possibility is that TraY, in conjunction with TraJ, also acts to antagonize H-NS repression at the P_Y promoter. Although the exact role of TraY in regulating P_Y and P_M is unclear, it appears to oppose H-NS-mediated repression at these promoters. Studies suggesting a role for TraY as a positive activator have been done with large promoter fragments (Silverman and Sholl, 1996), or with the F plasmid or its derivatives such as pOX38 (this work). Studies suggesting that TraY is a negative regulator (Taki *et al.*, 1998; this work), used smaller promoter fragments in transcriptional fusion constructs which lacked the upstream promoters, P_M and P_J. TraY-mediated activation of P_Y does not appear to be solely due to activation of P_M and subsequent transcriptional read-through of *traM* and *traJ*, as suggested in previous studies of the F-like plasmid, R100 (Stockwell *et al.*, 2000), since activation has been demonstrated in constructs lacking P_M (Silverman and Sholl, 1996). Thus, TraY could act either as an activator or repressor of P_Y depending on the DNA context within the promoter region on F plasmid. This context would be influenced by the nature of the repressor complex at P_Y, the degree of read-through transcription for the upstream promoters P_M and P_J, as well as by the superhelical density at P_Y, which is known to be responsive to supercoiling (Gaudin and Silverman, 1993). TraY could also act as an activator that aids TraJ in relieving H-NS repression and initiating transcription from P_Y. Alternatively, higher intracellular levels of TraY, present at later times in the growth cycle, or when supplied *in trans* or *in cis* during promoter assessment assays, could cause repression of the P_Y promoter either directly or by helping to establish the H-NS-based repressor complex. Several studies have demonstrated that all three transfer promoters, P_M, P_J, and P_Y, require relatively large segments of the flanking DNA for normal regulation (Gaudin and Silverman, 1993;

Silverman *et al.*, 1991b; Will *et al.*, 2004). One possible explanation for this is a requirement for transcriptionally-generated supercoiling at each of the promoters to provide the appropriate context for regulation. Another possibility is the formation of large gene-loops whereby the three main transfer region promoters are linked together through DNA-protein interactions and strand-bridging (Dame *et al.*, 2005). Since H-NS can bind to each of the transfer promoters, it might have a role in connecting these promoters into a single regulatory complex. This would allow for coordinate, cooperative regulation of all three promoters, and would explain why the examination of the transfer promoters in isolation results in puzzling or contradictory models for H-NS and TraY action (Will *et al.*, 2004).

The results reported here highlight the importance of H-NS, which, along with other host nucleoid-associated proteins, controls the expression of non-essential genes acquired by horizontal transfer. Because of its promiscuous binding activity, H-NS is able to repress a wide assortment of promoters, including those associated with mobile genetic elements, (Dorman, 2004; Hommais *et al.*, 2001). These elements appear to encode a number of proteins that can interact directly with H-NS and modulate or inhibit its activity. The F-like plasmids, R100 and pRK100, have acquired homologs to Hha, which are known to interact with H-NS (Nieto *et al.*, 2002; Starcic Erjavec *et al.*, 2003). In the case of R100, the Hha homolog, RmoA, has been shown to act as positive regulator of transfer (Nieto *et al.*, 1998). Several IncH plasmids have been shown to carry both H-NS and Hha homologs (Beloin *et al.*, 2003; Gilmour *et al.*, 2004), which appear to play a role in regulating plasmid transfer gene expression (Forns *et al.*, 2005). We are currently investigating the mechanism of TraJ antagonism of H-NS to determine whether it occurs

through binding DNA to disrupt a repressor complex or by binding H-NS directly thereby affecting its activity.

Chapter 5: Hfq is a regulator of F plasmid TraJ and TraM synthesis*

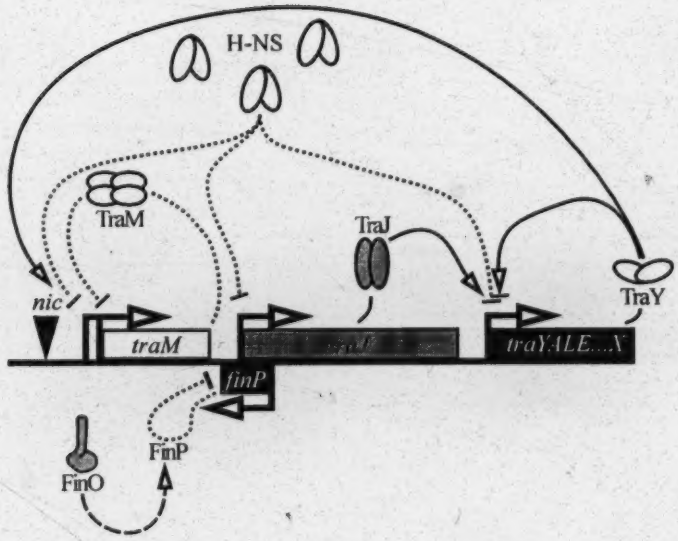
*Portions of this chapter were published: Will, W.R., and Frost, L.S., (2006) *J Bacteriol.* **188**: 124-131.

5.1 Introduction

The F plasmid of *Escherichia coli* is considered a model for bacterial conjugation, and has been studied extensively (Gubbins *et al.*, 2005). F plasmid transfer is facilitated by a large multi-component protein complex which spans the donor cell membrane to transport plasmid DNA into a recipient cell. This complex is encoded by a single 33 kb polycistronic transfer (*tra*) operon (Frost *et al.*, 1994). Recent studies have demonstrated that the regulatory circuit controlling the expression of this *tra* operon is complex. However, the focal point of control appears to be TraJ. TraJ is the primary activator of *tra* operon expression, encoded on a monocistronic operon immediately upstream of the *tra* operon and downstream from *traM* (Willetts, 1977; Frost *et al.*, 1994) (Fig. 5.1). It appears to act by opposing H-NS-mediated repression of the *tra* operon promoter, P_Y. The resulting derepression of P_Y allows the synthesis of the secondary regulator, TraY, encoded by the *tra* operon, which further activates P_Y, as well as *traM* (Penfold *et al.*, 1996). TraM is required to transmit the signal for the initiation of DNA transfer between the transfer complex and the relaxosome, which nicks and unwinds the plasmid DNA during transfer (Disque-Kochem and Dreiseikelmann, 1997; Lu and Frost, 2005). TraM also has a regulatory role as an autorepressor that represses expression from the two tandem *traM* promoters, referred to here collectively as P_M (Penfold *et al.*, 1996).

In most F-like plasmids, TraJ synthesis is subject to post-transcriptional control via the FinOP antisense RNA system, whereby *finP* encodes a small anti-sense RNA complementary to the 5' untranslated region (UTR) of the *traJ* transcript (van Biesen and Frost, 1994). FinP is a short, 79 base RNA consisting of two stem-loop sequences, SL-I

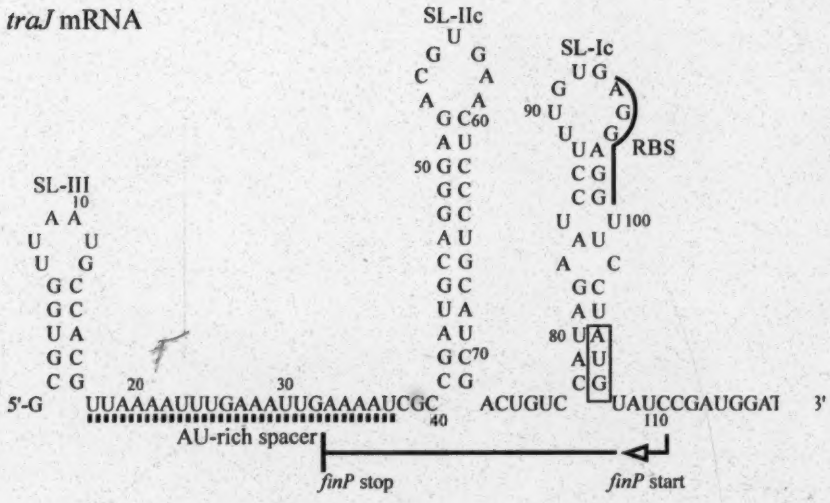
Figure 5.1 The F plasmid regulatory circuit is composed of three major plasmid-encoded regulatory proteins: TraJ, TraY, and TraM. TraJ is the primary activator, and appears to act by countering repression by the host factor, H-NS, at the *tra* operon promoter, P_Y. TraY also activates *tra* gene at P_Y, as well as the *traM* promoter, P_M. TraM and TraY act as autorepressors at their respective promoters with TraY also having a role as an activator in exponential phase. Most F-like plasmids are also regulated by the FinOP antisense RNA system, where an antisense RNA, FinP, complementary to the 5' UTR of the *traJ*, forms a duplex with that transcript and sequesters the *traJ* RBS. FinO, an RNA chaperone encoded by the plasmid, is necessary to stabilize FinP and promote duplex formation. However, *finO* is disrupted in the F plasmid, resulting in the derepression of *tra* gene expression.


H-NS
TraM
TraJ
TraY
nic
traM
traYALE....X
finP
FinP
FinO

and SL-II (van Biesen *et al.*, 1993) (Fig. 5.2). These are complementary to stem-loops present in the 5' UTR of *traJ*, referred to as SL-Ic and SL-IIc, respectively. A third stem-loop, SL-III, is also present in the 5' UTR of the *traJ* mRNA, but not in FinP, and is separated from SL-IIc by a 24 base AU-rich spacer region. These structural elements appear to be well conserved, and are present in most F-like *traJ* transcripts (Frost *et al.*, 1994). FinO, a plasmid-encoded RNA chaperone, binds FinP, protecting it from degradation by RNase E and promoting duplex formation between the complementary stem-loop structures in both FinP and *traJ* (Jerome and Frost, 1999; Arthur *et al.*, 2003). The resulting duplex sequesters the ribosome binding site (RBS) and prevents the translation of *traJ*, resulting in the repression of plasmid transfer (Koraimann *et al.*, 1996; Gubbins *et al.*, 2003). However, the F plasmid is naturally derepressed since its copy of *finO* has been disrupted by the insertion of an IS*3* element (Cheah and Skurray, 1986).

In this study, we considered the possibility that host proteins might also affect *traJ* mRNA synthesis using a post-transcriptional mechanism similar to the FinOP system in F-like plasmids, with Hfq, the host RNA chaperone, being the most likely candidate. Originally characterized as Host Factor I (HF-I), a host-encoded protein necessary for the *in vitro* replication of the Qβ RNA bacteriophage (Franze de Fernandez *et al.*, 1968), Hfq has emerged as a potent regulator of many aspects of RNA biology, influencing stability, translation, and RNA bacteriophage replication, often via small RNAs (Gottesman, 2004; Valentin-Hansen *et al.*, 2004). Hfq is an 11.2 kDa protein, forming hexamers that preferentially bind sequences of AU-rich RNA, often flanked by structured regions (Møller *et al.*, 2002; Zhang *et al.*, 2002). It is relatively abundant, and is present at intracellular levels of approximately 10,000 hexamers per cell (Ali Azam *et*

Figure 5.2 The secondary structure of *traJ*. The 5' UTR of the *traJ* mRNA contains an AU-rich spacer, indicated by a dashed line, to which Hfq binds. The complementary antisense RNA, FinP, encoding SLI and SLII, contains only a portion of the complementary sequence of the spacer between SLIIc and SLIII in *traJ* mRNA. The transcriptional start and stop sites for FinP are indicated beneath the *traJ* transcript. The transcript is numbered relative to its 5' terminus. The RBS is indicated by a solid line, and the start codon is boxed.


traJ mRNA
SL-IIc
SL-Ic
SL-III
RBS
5'-G
UUAAAAUUUGAAAUUGAAAAUCGC
ACUGUC
UAUCCGAUGGAT
3'
AU-rich spacer
finP stop
finP start

al., 1999). However, the expression profile throughout the growth cycle remains uncertain, as some studies have indicated that Hfq levels peak and then decrease rapidly during lag phase, dropping to approximately one third of the intracellular maximum throughout the rest of the growth cycle (Ali Azam *et al.*, 1999). Other studies have found that Hfq levels increase as cultures enter stationary phase or during periods of slow growth (Tsui *et al.*, 1997; Vytvytska *et al.*, 1998).

Given that TraJ synthesis is regulated by both an antisense RNA system, a common target of Hfq, and H-NS, which has been shown to overlap with Hfq in many regulatory circuits, including *rpoS*, *hns*, and *bgl* regulation (Sledjeski and Gottesman, 1995; Hengge-Aronis, 1996; Lease *et al.*, 1998; Sledjeski *et al.*, 2001; Dole *et al.*, 2004), it seemed possible that Hfq might target and regulate TraJ mRNA in some manner. This study demonstrates that Hfq binds to the intergenic UTR, 3' to *traM* and 5' to *traJ*, and decreases the stability of transcripts containing this region. Hfq does not appear to be involved in fertility inhibition and has no role in FinOP-mediated repression. Instead, Hfq appears to act as a repressor of TraJ and TraM synthesis, as well as F plasmid transfer in general, by destabilizing the corresponding transcripts.

5.2 Results

5.2.1 Hfq represses F plasmid transfer as donor cell cultures enter stationary phase

To examine the effect of Hfq on F plasmid transfer, two mutant host strains were used. The first mutant strain, AM111, contains an *hfq1*::Ω mutation, which is located near the 5' terminus of the *hfq* gene, disrupting it and resulting in an *hfq*$^-$ phenotype (Tsui *et al.*, 1994). To assay for possible downstream polar effects, a second strain, AM112, was used. AM112 contains the *hfq2*::Ω mutation, located near the 3' terminus of the *hfq*

gene, which allows for functional Hfq synthesis while still disrupting transcription of the downstream genes (Tsui *et al.*, 1994). In these analyses of the F plasmid, AM112 behaved identically to the wild-type isogenic strain, MC4100, and was used as such (data not shown).

AM111 and AM112 containing pOX38-Tc, an F plasmid derivative containing the entire transfer region, were grown to stationary phase, and at the indicated time-points, samples were taken and assayed for mating efficiency (Fig. 5.3). The results indicated that Hfq has a growth phase-dependent, negative effect on plasmid transfer. Mating efficiency in AM112 decreased rapidly as the culture approached stationary phase, as was observed in the wild-type strain, MC4100 (Will *et al.*, 2004). However, the *hfq*⁻ donor strain, AM111, exhibited a slower and smaller decrease in mating efficiency than AM112, with the difference between the two strains becoming more pronounced as the cultures entered stationary phase. There was a slight difference in growth rate between the two strains, but this was discounted as a factor since there was a significant difference in mating efficiency after 24 hours of growth. Mating efficiency was also assayed in donor strains containing pSnO104, which provides functional FinO *in trans*, restoring FinOP-mediated repression of TraJ synthesis. Mating was fully repressed in both AM111 and AM112, suggesting that Hfq was not necessary for FinOP-mediated repression (Table 5.1).

5.2.2 Stationary phase TraJ and TraM levels are increased in an *hfq* host

Since Hfq could be influencing plasmid transfer by altering the expression of the three regulators, TraM, TraJ and TraY, their respective transcripts were examined for

Figure 5.3 F plasmid transfer is increased in stationary phase in *hfq1*::Ω (AM111) host cells. Standing overnight AM112/pOX38-Tc and AM111/pOX38-Tc donor cultures were diluted into fresh media, and at the indicated time-points, samples were removed and mated with ED24 in spent media to determine the mating efficiency of either strain at that time-point. Data for AM111/pOX38-Tc is indicated by a dashed line, whereas data for AM112/pOX38-Tc is indicated by a solid line. Mating efficiency is indicated by squares, whereas OD_{600} is indicated by triangles.

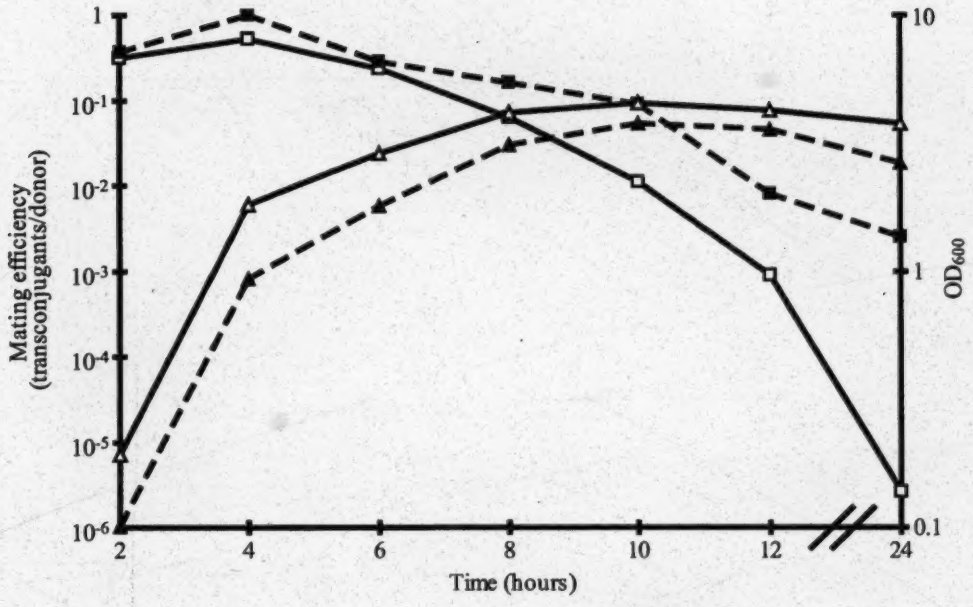

1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Mating efficiency
(transconjugants/donor)
10
1
0.1
OD_{600}
2
4
6
8
10
12
24
Time (hours)

Table 5.1 Hfq is not required for FinOP-mediated repression of F plasmid transfer.

Donor strain	Mating efficiency (transconjugants/donor)
AM112/F*lac*	0.18
AM112/F*lac*/pSnO104	2.6×10^{-3}
AM111/F*lac*	0.69
AM111/F*lac*/pSnO104	7.6×10^{-4}

putative Hfq binding sites. Only one putative binding site was identified, which was located in the *traM-traJ* intergenic region (Fig. 5.2). The site consisted of the 24 base AU-rich spacer flanked by stem-loops SL-III and SL-IIc in the 5' *traJ* mRNA UTR, which was similar to many other documented Hfq binding sites. However, this sequence is absent from the complementary antisense RNA, FinP. Whereas this UTR is present at the 5' terminus of the *traJ* mRNA, it is also present at the 3' terminus of the *traM* transcript, potentially acting as a rho-independent transcriptional terminator. Thus, it seemed likely that the *traM* and *traJ* transcripts were targets of Hfq.

To determine if this was the case, immunoblot analysis was performed on AM111/pOX38-Tc and AM112/pOX38-Tc donor cell cultures in exponential phase growth (at 0.5 OD_{600}, after approximately 2.5 hours of growth), early stationary phase (8 hours), and late stationary phase (24 hours), to determine the intracellular levels of the three plasmid regulators (Fig. 5.4). In exponential phase, only TraJ levels varied between the two strains, appearing to be slightly lower in AM111/pOX38-Tc. However, after both 8 and 24 hours of growth, the intracellular levels of both TraJ and TraM were present at higher levels in AM111/pOX38-Tc compared to AM112/pOX38-Tc. However, TraY levels did not differ between the two strains at any time point. This suggests that the increase in TraM levels was not due to an increase in TraY because of an indirect effect of TraJ. Rather, it implies that Hfq acted directly on both *traM* and *traJ* mRNA. TraM and TraJ levels were also assayed in both donor strains containing pSnO104, providing FinO *in trans*. The mutation of *hfq* did not have any effect on FinOP-mediated repression, further suggesting that Hfq acts independently of FinOP (Figure 5.5).

Figure 5.4 Stationary phase TraJ and TraM levels are increased in *hfq1*::Ω (AM111) host cells. To determine the intracellular levels of the three plasmid regulators, TraJ, TraM, and TraY, standing overnight cultures of AM111/pOX38-Tc and AM112/pOX38-Tc were diluted into fresh LB broth and at the appropriate time-points, samples were collected for immunoblot analysis. Protein levels were assayed in exponential phase (at 0.5 OD_{600}, after approximately 2.5-3 hours of growth), stationary phase (8 hours of growth), and late stationary phase (24 hours of growth). A non-specific band used as a loading control is also indicated.

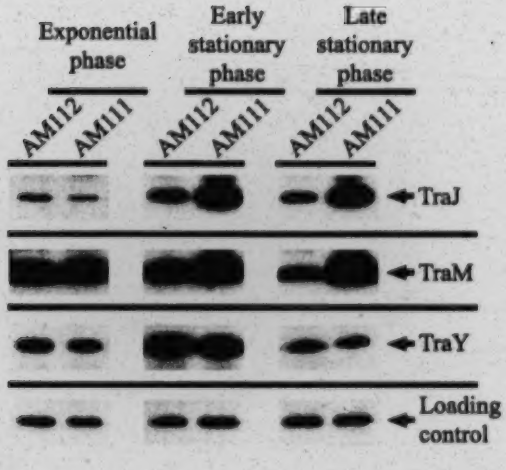
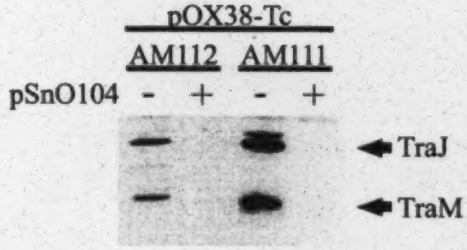

Exponential phase
Early stationary phase
Late stationary phase
AM112
AM111
AM112
AM111
AM112
AM111
TraJ
TraM
TraY
Loading control

Figure 5.5 Hfq is not necessary for FinOP-mediated repression of TraJ synthesis. To determine whether Hfq is necessary for FinOP activity, TraJ and TraM levels were assayed in AM112/pOX38-Tc and AM111/pOX38-Tc donor strains containing pSnO104, which provides FinO *in trans*. Cultures were grown for 24 hours, into stationary phase, before samples were taken.


pOX38-Tc
AM112
AM111
pSnO104 - + - +
TraJ
TraM

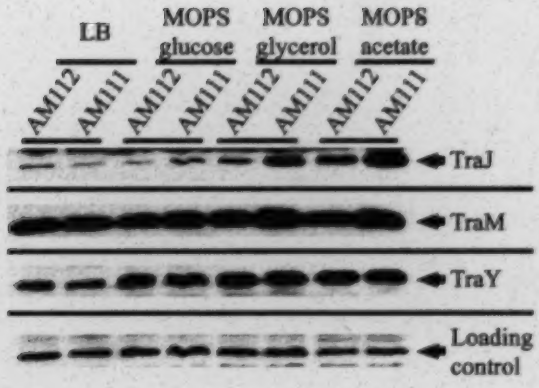
5.2.3 Hfq-mediated growth rate-dependent regulation of TraJ levels

As Hfq has been demonstrated to regulate *ompA* mRNA stability and translation in a growth rate-dependent manner, the effect of an altered growth rate on Hfq-mediated control of TraJ, TraM, and TraY synthesis was also examined. AM111/pOX38-Tc and AM112/pOX38-Tc cultures were grown to exponential phase in LB broth or minimal MOPS media containing glucose, glycerol, or acetate as a carbon source and examined via immunoblotting for TraJ, TraM, and TraY levels (Figure 5.6). While TraJ levels appeared to be slightly lower in AM111 than in AM112 when grown in LB, the opposite occured when grown in MOPS media, particularly on the poorer carbon sources, glycerol and acetate. Although, TraJ levels were higher in AM111 than in AM112 in the synthetic minimal media, there was only a slight increase visible in TraM levels on glycerol and acetate, and TraY levels did not fluctuate at all. It is also interesting to note that the overall TraJ levels increase as the growth rate decreases in both AM111 and AM112. The reason for this is unknown, but may be due to regulation by CRP or other metabolically sensitive factors.

5.2.4 Hfq binds the *traM-traJ* intergenic UTR

To determine if Hfq binds the *traM-traJ* intergenic UTR, electrophoretic mobility shift assays were performed with pure Hfq protein and *in vitro* synthesized RNA templates (Fig. 5.2). As the complementary FinP lacks most of the complementary spacer sequence, as well as any sequence complementary to SL-III, Hfq was predicted not to bind to FinP, or do so with a much lower affinity. Binding assays were performed with *traJ*184 RNA, which consists of the first 184 bases of the *traJ* transcript, including the predicted binding site, and FinP RNA. In the absence of competitor, Hfq bound both

Figure 5.6 Hfq-mediated repression is growth rate-dependent. To determine whether Hfq-mediated repression of the F plasmid is growth-rate dependent, the intracellular levels of TraJ, TraY, and TraM were determined. Standing overnight cultures were diluted into fresh media, either LB broth or MOPS media supplemented with glucose, glycerol, or acetate as a carbon source. Cultures were then grown with shaking at 37°C to 0.5 OD_{600}, and samples were collected for immunoblot analysis. A non-specific band used as a loading control is also shown. Results are representative of triplicate experiments.


LB
MOPS glucose
MOPS glycerol
MOPS acetate
AM112
AM111
TraJ
TraM
TraY
Loading control

fragments, although its affinity for *traJ*184 (Fig. 5.7A) was significantly higher than that for FinP (Figure 5.7B). The binding assays were then repeated in the presence of an excess of tRNA, acting as a competitor, to demonstrate specific binding. Hfq bound *traJ*184 in the presence of the competitor (Fig. 5.7C) with a dissociation constant of K_d=83 nM. However, binding to FinP was almost completely inhibited by the presence of tRNA (Fig. 5.7D), suggesting that Hfq binds the predicted site in the *traJ* UTR specifically.

5.2.5 Plasmid transcriptional profiles are altered in an *hfq* mutant host

In wild-type donor strains, transcription of *traM* and *traJ* is highest early in exponential phase, and then decreases rapidly as cells progress towards stationary phase (Will *et al.*, 2004). If Hfq was influencing transcript levels, likely by targeting the *traJ* and *traM* mRNA for degradation, transcript levels would be predicted to increase as the donor culture approached stationary phase in AM111 when compared to AM112. To determine if Hfq directly alters transcript levels, or inhibits translation, samples were collected from AM111/pOX38-Tc and AM112/pOX38-Tc donor cultures at regular intervals throughout the growth curve and total cellular RNA was extracted and examined by northern blot analysis. Blots were probed with *in vitro* synthesized [^{32}P]-labeled FinP RNA to detect *traJ* mRNA (Fig. 5.8A). AM112/pOX38-Tc displayed a wild-type transcriptional profile, with *traJ* transcripts decreasing as the donor cultures progressed towards stationary phase, becoming nearly undetectable after 8 hours of growth. AM111/pOX38-Tc had a very different profile. Transcript levels were lower than wild-type at the earliest time-points, but slowly accumulated and peaked after 6 hours of growth. The initial lag in transcript levels can be explained by secondary effects via

Figure 5.7 Hfq specifically binds the *traJ* 5' UTR. Electrophoretic mobility shift analysis was performed to analyze the binding affinity of Hfq for the *traJ* 5' UTR and FinP RNA. *In vitro* transcribed ^{32}P-labelled *traJ*184 (A) and FinP (B) RNA was incubated in the presence of increasing concentrations of Hfq, and the resulting Hfq-RNA complexes were then resolved by electrophoresis in a 6% TBE-polyacrylamide gel. To demonstrate specific binding activity, Hfq was incubated with *traJ*184 (C) and FinP (D) RNA in the presence of 100 μg/ml *E. coli* tRNA. Unbound RNA species are indicated adjacent to each gel. The concentration of Hfq present in each reaction is indicated above its respective lane.

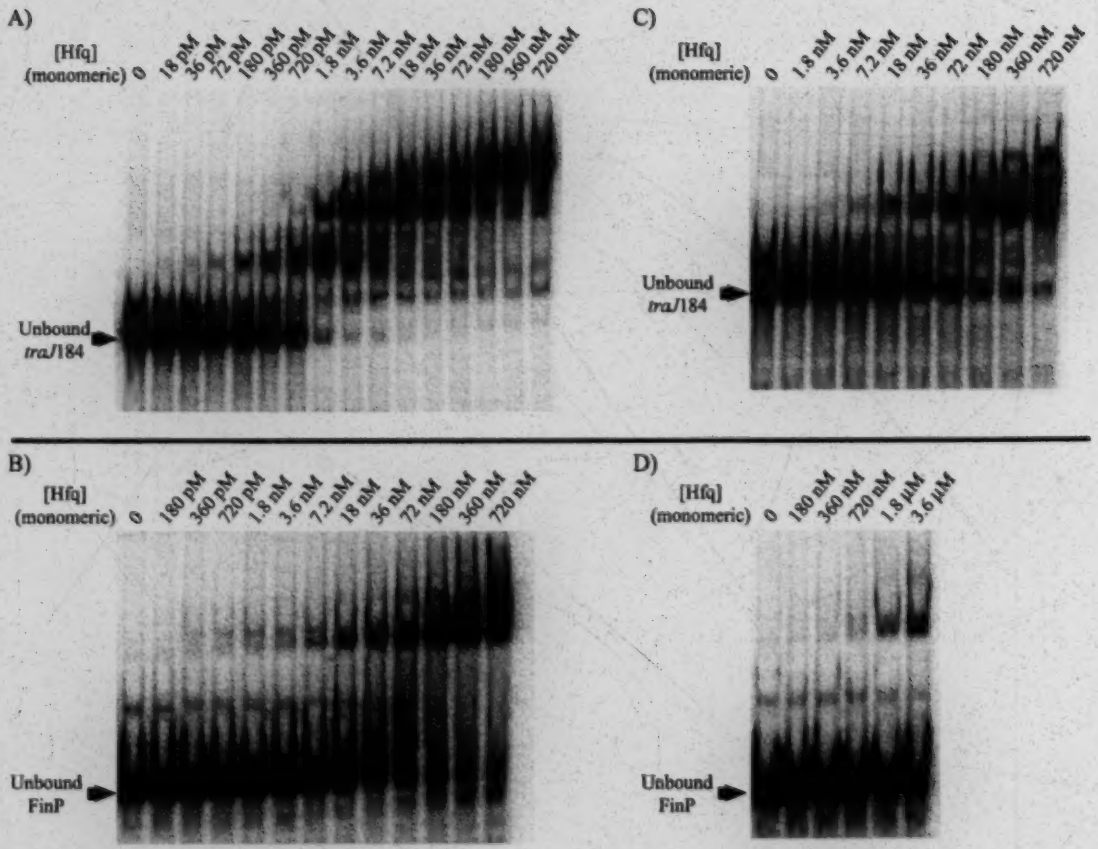
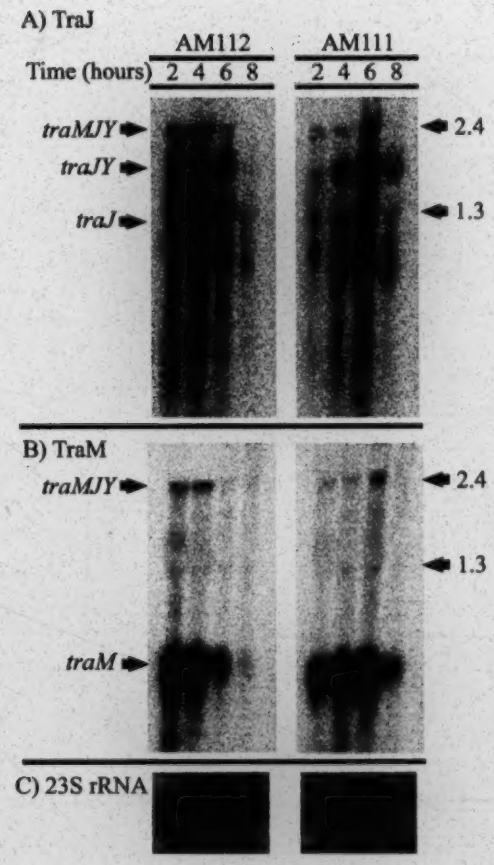

A)
[Hfq] (monomeric)
0
18 pM
36 pM
72 pM
180 pM
360 pM
720 pM
1.8 nM
3.6 nM
7.2 nM
18 nM
36 nM
72 nM
180 nM
360 nM
720 nM
Unbound tra/184
C)
[Hfq] (monomeric)
0
1.8 nM
3.6 nM
7.2 nM
18 nM
36 nM
72 nM
180 nM
360 nM
720 nM
Unbound tra/184
B)
[Hfq] (monomeric)
0
180 pM
360 pM
720 pM
1.8 nM
3.6 nM
7.2 nM
18 nM
36 nM
72 nM
180 nM
360 nM
720 nM
Unbound FinP
D)
[Hfq] (monomeric)
0
180 nM
360 nM
720 nM
1.8 µM
3.6 µM
Unbound FinP

Figure 5.8 *traJ* and *traM* transcript profiles are altered in an *hfq1*::Ω (AM111) host strain. To determine the effect of Hfq on *traJ* and *traM* transcript profiles, total cellular RNA was collected from AM111/pOX38-Tc and AM112/pOX38-Tc donor cultures at the time-points indicated above each sample and analyzed by northern hybridization. Membranes were probed for *traJ* mRNA (A), using ^{32}P-labelled FinP RNA, or *traM* mRNA (B), using ^{32}P-end-labelled SPE5-ext. The relative positions of two molecular weight marker bands (2.4 kb and 1.3 kb) are indicated. Transcript species are designated *traMJY*, *traJY*, *traJ*, and *traM*, based on their predicted identities. Membranes were examined for loading and transfer efficiency using ^{32}P-end-labelled 23SR3, which hybridizes specifically with the 3' terminus of 23S rRNA (C).


A) TraJ
AM112
AM111
Time (hours)
2 4 6 8
2 4 6 8
traMJY
traJY
traJ
2.4
1.3
B) TraM
traMJY
2.4
1.3
traM
C) 23S rRNA

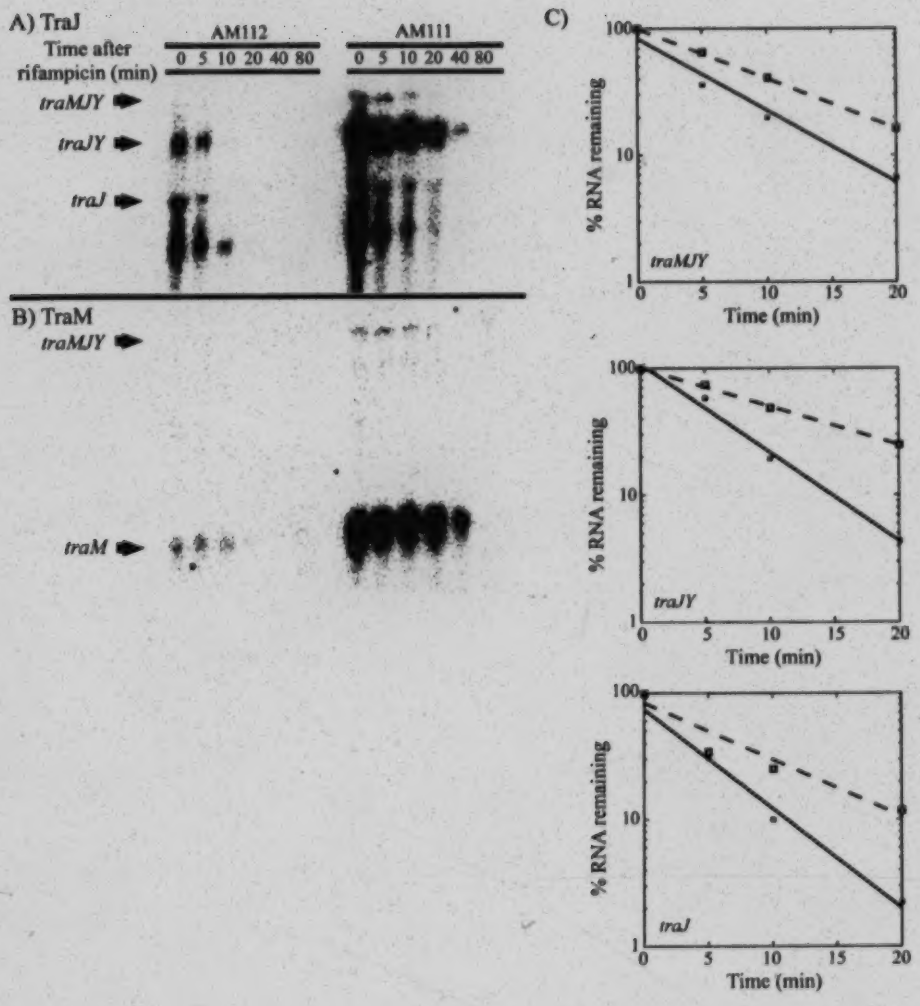
increased H-NS levels in AM111(Sledjeski *et al.*, 2001), and hence, increased H-NS-mediated repression of *traJ* transcription (see section 5.3). Despite the increase in H-NS-mediated repression, transcripts accumulated and remained detectable in stationary phase, likely due to increased stability. Transcripts larger than the predicted *traJ* transcript (which is approximately 900 bases) were attributed to transcriptional read-through, both from the upstream *traM* promoter, P_M, and from P_J, driving transcription downstream into *traY*. These larger transcripts include *traMJY*, a 2.4 kb transcript which is thought to be transcribed from P_M, and *traJY*, a 1.8 kb transcript which is thought to be transcribed from P_J (Will *et al.*, 2004). Both of these transcripts should carry the Hfq binding site: *traMJY* should carry the site in the middle of the transcript, between *traM* and *traJ*, whereas *traJY* should carry the site at its 5' terminus. The transcription profile for these read-through products was also altered, peaking after 6 hours of growth. Blots were stripped and re-probed with [^{32}P]-end-labeled SPE5-ext, which detects *traM*-containing transcripts (Fig. 5.8B). As with *traJ* mRNA, transcript levels appeared to peak after approximately 6 hours of growth, and were still readily detectable after 8 hours. The increased transcript levels present at later time points suggest that despite increased transcriptional repression via H-NS, Hfq negatively controls *traJ* and *traM* transcript levels, possibly by altering transcript stability.

5.2.6 Transcript stability is increased in *hfq* mutant host cultures

To determine whether Hfq influences *traJ* and *traM* transcript stability, possibly via the binding site in the *traM-traJ* intergenic region, we examined the half-life of RNA species detected by probes specific for both *traJ* and *traM* in the *hfq1*::Ω host strain, AM111. AM111/pOX38-Tc and AM112/pOX38-Tc donor cell cultures were grown in

LB broth, transcription was then inhibited by the addition of rifampicin, and total cellular RNA was collected at the indicated time-points. RNA was then used in northern blot analysis and probed with *in vitro* synthesized, [^{32}P]-labeled FinP RNA, specific to the 5' UTR of the *traJ* transcript (Fig. 5.9A), and [^{32}P]-end-labeled SPE5-ext, which is specific to *traM* (Fig. 5.9B). The half-life of the *traMJY* read-through transcript increased from approximately 4 minutes in AM112 to 8 minutes in AM111. This suggests that Hfq is destabilizing the *traMJY* transcript, thereby promoting its degradation. The second read-through transcript, *traJY*, also increased in stability in AM111. The half-life of *traJY* was 4.8 minutes in AM112, which increased to approximately 10 minutes in AM111. The stability of the smaller monocistronic *traJ* transcript was also affected by Hfq (Fig. 5.9C), but its signal was obscured by what appeared to be degradation products that migrated to the same position, preventing an accurate determination of half-life. The smaller fragments migrating below *traJ* are thought to be degradation products. Their stability did not appear to be affected by Hfq. Accurate half-life determination of the monocistronic *traM* transcripts was not possible because the appropriate probe detected two closely migrating bands, the smaller of which was a degradation product that cannot be resolved from the *traM* mRNA, under these experimental conditions (Will *et al.*, 2004). Transcript stabilities do not appear to be altered in a growth phase-dependent manner, as similar half-lives were observed after both 3 and 6 hours of growth (data not shown). Nonetheless, Hfq appears to decrease the stability of *traM* and *traJ* transcripts containing the Hfq binding site.

Figure 5.9 *traJ* and *traM* transcript stability is altered in an *hfq1*::Ω (AM111) host strain. After 6 hours of growth, 200 μg/ml of rifampicin was added to AM111/pOX38-Tc and AM112/pOX38-Tc cultures to halt further transcription. At the indicated time-points, culture samples were removed and total cellular RNA was extracted. RNA samples were then analyzed by northern hybridization to determine transcript half-lives. Blots were probed for *traJ* containing transcripts (A) using ^{32}P-labelled FinP RNA, or *traM* containing transcripts (B) using ^{32}P-end-labelled SPE5-ext. Transcript species are designated *traMJY*, *traJY*, *traJ*, and *traM*, based on their predicted identities. The relative positions of two molecular weight marker bands (2.4 kb and 1.3 kb) are indicated. The relative amounts of each of the species were determined using ImageQuaNT software (GE Healthcare). Relative signal intensities were plotted against time for each of the major fragments in both AM111/pOX38-Tc (white squares) and AM112/pOX38-Tc (black squares) (C). Stability data for the smaller degradation products are not shown.


A) TraJ
AM112
AM111
Time after rifampicin (min)
0 5 10 20 40 80
0 5 10 20 40 80
traMJY
traJY
traJ
B) TraM
traMJY
traM
C)
% RNA remaining
100
10
1
0 5 10 15 20
Time (min)
traMJY
traJY
traJ

5.3 Discussion

The data presented in this study indicate that the host Sm-like protein, Hfq, regulates F plasmid transfer by repressing the synthesis of the plasmid regulators, TraJ and TraM by binding to an AU-rich UTR encoded between *traM* and *traJ* and destabilizing the corresponding transcripts. Hfq is a global RNA chaperone that can regulate genes post-transcriptionally by modulating both transcript stability and translational initiation, either positively or negatively (Gottesman, 2004). In the case of the F plasmid, Hfq appears to destabilize multiple transcripts that share a single Hfq binding site located in the *traM-traJ* intergenic UTR (Fig. 5.2). However, despite the fact that Hfq often regulates other systems via small RNAs (Zhang *et al.*, 2002; Masse *et al.*, 2003), it does not appear to be involved in the FinOP antisense RNA system, which represses *traJ* translation in F-like plasmids. This suggests that Hfq-mediated regulation of transfer gene expression occurs by a distinct mechanism.

Analysis of mating efficiency in both wild-type (AM112) and *hfq1*::Ω (AM111) donor cultures demonstrated increased mating ability in *hfq1*::Ω cultures as they entered stationary phase. Similarly, the intracellular levels of two of the three plasmid transfer regulatory proteins, TraJ and TraM, were increased in *hfq1*::Ω donor cultures as they entered stationary phase. However, there was no difference in intracellular TraY levels in wild-type and *hfq1*::Ω donor cultures at any point during the growth curve. TraJ levels were also increased in *hfq1*::Ω strains grown on minimal media, resulting in slower growth rates. This suggested that Hfq influenced F plasmid transfer by affecting *traM* and *traJ* mRNA. Furthermore, these results indicated that Hfq-mediated repression of

TraM was direct, since TraY, an essential activator of *traM* transcription, was unaffected by Hfq (Penfold *et al.*, 1996).

Hfq typically binds stretches of AU-rich RNA, often flanked by regions with significant secondary structure (Møller *et al.*, 2002; Zhang *et al.*, 2002). Therefore, the transcript sequences for the three F plasmid regulatory genes, *traJ*, *traM*, and *traY*, were examined for putative Hfq binding sites. One site was identified, located in the *traM-traJ* intergenic UTR (Fig. 5.2). This sequence is located at the 5' terminus of transcripts originating from P_J, the *traJ* promoter, as well as at the 3' terminus of *traM* transcripts, and in the middle of read-through transcripts from P_M. This sequence is also critical for duplex formation with FinP, the complementary antisense RNA, which represses translation of *traJ* (Koraimann *et al.*, 1996; Gubbins *et al.*, 2003). Electrophoretic mobility shift analysis demonstrated that Hfq bound *traJ* RNA containing the putative binding site with specificity. However, FinP, which lacks most of the AU-rich spacer, was not bound by Hfq in a physiologically relevant manner.

Transcriptional analysis of F plasmid *tra* gene expression in the *hfq1*::Ω (AM111) and wild-type (AM112) strains via northern blotting presented an intriguing profile. Whereas transcription of *tra* genes peaked early in exponential growth in a wild-type host, and then rapidly decreased as the culture progressed towards stationary phase, reaching undetectable levels (Will *et al.*, 2004), transfer gene transcript levels in *hfq1*::Ω mutant hosts were lower in early exponential phase, and peaked in late exponential phase. *traJ* and *traM* transcript levels were still detectable in *hfq1*::Ω donor cultures in early stationary phase, albeit at lower than maximal levels. This delayed transcript peak is thought to be due to increased H-NS levels, as H-NS has been shown to be a potent

repressor of *tra* gene expression (Will *et al.*, 2004). Hfq is critical in facilitating repression of H-NS synthesis by the small RNA, DsrA, originally described as an H-NS antisilencer (Sledjeski and Gottesman, 1995; Sledjeski *et al.*, 2001). Disruption of *hfq* results in partial activity of DsrA, leading to increased H-NS and H-NS-mediated repression of the *tra* genes and other H-NS targets. This could explain the lower levels of *traJ* and *traM* transcription observed in exponential phase in AM111. However, *tra* gene expression is still sufficient in the *hfq1*::Ω (AM111) host to support normal transfer levels. Analysis of transcript stability indicated that *traJ* and *traM*-containing transcripts were stabilized in an *hfq1*::Ω host. Transcript half-lives, particularly for the larger transcripts, increased approximately 2-fold in the *hfq1*::Ω donor strain. An increase in transcript stability would allow for transcript accumulation, resulting in increased transcript levels at later time points. These results suggest that Hfq promotes the degradation of *traJ* and *traM* transcripts by an unidentified nuclease. The destabilization of multiple transcripts does not require multiple Hfq binding sites, but appears to utilize a single binding region located in the *traJ-traM* intergenic UTR, independent of its position within the transcript. Thus, Hfq binding could alter the secondary structure of the UTR so as to promote cleavage by endoribonucleases, in particular RNaseE, which attacks AU-rich sequences, and is involved in the degradation of FinP (Jerome *et al.*, 1999), and is commonly involved in Hfq-mediated control of transcript stability (Masse *et al.*, 2003; Moll *et al.*, 2003a). Alternatively, Hfq might inhibit ribosome binding, as described in the case of the *ompA* transcript, which then allows for more efficient degradation by RNaseE (Vytvytska *et al.*, 2000).

Despite its well documented role in many antisense control systems (Gottesman, 2004), Hfq does not appear to be involved in FinOP control of TraJ synthesis. It seemed plausible that Hfq, given its role in other systems as an RNA chaperone (Geissmann and Touati, 2004), might serve to promote duplex formation between FinP and the *traJ* 5' UTR, and that FinOP-mediated repression of F might also require Hfq as a cofactor. Although this study does not indicate a role for Hfq in the repression of TraJ synthesis by the antisense RNA, FinP, it is possible that Hfq-mediated destabilization of *traM* and *traJ* mRNA requires the presence of a small RNA, encoded by the F plasmid or the host. However, the *traJ* 5' UTR, including the AU-rich spacer, is well conserved in F-like plasmids (Frost *et al.*, 1994), suggesting that Hfq-mediated control of transfer gene expression is common amongst these plasmids. This suggests that Hfq is important in destabilizing *traM* and *traJ* transcripts to promote a rate of turnover which is in balance with the transfer potential of F donor cells, allowing for greater sensitivity to transcriptional cues from the environment.

Chapter 6: The effect of nucleoid-associated proteins on F plasmid transfer: a general activational role for IHF

6.1 Introduction

Bacterial conjugation allows the transfer of DNA from a donor cell to a recipient cell by way of a multi-component protein complex. Conjugative plasmids can carry any number of genes, involved in a wide array of cellular functions, such as antibiotic resistance, catabolism, and pathogenesis. As a result, conjugative plasmids are extremely diverse. However, the F plasmid of *Escherichia coli*, discovered nearly 60 years ago (Lederberg and Tatum, 1946), is likely the most understood. The F plasmid is 100 kb in size, with nearly all of the genes necessary for transfer encoded by a single 33 kb polycistronic transfer (*tra*) operon (Frost *et al.*, 1994). In addition, there are two monocistronic operons immediately upstream of the *tra* operon promoter, *traJ* and *traM*, which are also necessary for transfer.

The regulatory circuit controlling *tra* gene expression appears to be extremely complex, consisting of both plasmid and host-encoded factors. H-NS, the host nucleoid-associated protein, binds to the *traM*, *traJ*, and *tra* operon promoters, P_M, P_J, and P_Y, respectively, and represses transcription (Will *et al.*, 2004). TraJ, the primary activator, opposes H-NS silencing at P_Y. This allows the expression of the *tra* operon, including *traY*, the first gene in the operon. TraY acts as a secondary regulator to the system, regulating *tra* operon expression (Silverman and Sholl, 1996; Taki *et al.*, 1998), as well as activating transcription of *traM* (Penfold *et al.*, 1996). F plasmid transfer gene expression is also subject to post-transcriptional control via the host-encoded RNA binding protein, Hfq, and by the FinOP antisense RNA system in related F-like plasmids (Frost *et al.*, 1994). However, the system has been inactivated in F due to the insertion of an IS*3* element in the *finO* gene (Cheah and Skurray, 1986), although recent studies

suggest that *finP* is still required for normal regulation of the F plasmid regulatory circuit (Will *et al.*, 2004).

The expression of non-essential genes, like those carried by mobile elements such as the F plasmid, is often subject to physiologically-dependent control by the host cell. This type of regulation is often mediated by global regulatory proteins, including host histone-like proteins, which are thought to play important roles in organizing bacterial DNA (Dorman and Deighan, 2003). This group includes IHF, HU, Fis, H-NS, and its paralogue, StpA. More sequence-specific regulatory proteins like Lrp and CRP, as well as the RNA-binding protein, Hfq, are also often involved in global gene regulation. Recent studies have identified roles in IncF plasmid regulation for H-NS (Will *et al.*, 2004), Crp (Starcic *et al.*, 2003), Lrp (Camacho and Casadesus, 2002), and Hfq (see Chapter 5). In this study, the remaining regulatory proteins, HUα, HUβ, Fis, IHF, and StpA, are screened for any effect on F plasmid transfer ability, or a regulatory effect on the expression of the three plasmid regulators, TraJ, TraY, and TraM.

Fis is an 11.2 kDa protein first described for its role in promoting recombination by the Hin family recombinase (Kahmann *et al.*, 1985; Johnson *et al.*, 1986; Johnson *et al.*, 1988). It exhibits a dynamic expression profile, peaking in early exponential phase, at intracellular levels of almost 60,000 molecules per cell, then rapidly decreasing, reaching nearly undetectable levels in stationary phase (Ali Azam *et al.*, 1999). As a result, it is capable of dynamic, growth phase-dependent gene expression. Although its role in the regulation of rRNA synthesis is likely the best characterized (Schneider *et al.*, 2003), its regulatory effects are far-reaching. Fis has been shown to be involved in the regulation of many systems, including DNA gyrase synthesis (Schneider *et al.*, 2000;

Keane and Dorman, 2003), *rpoS* (Hirsch and Elliott, 2005), *hns* (Falconi *et al.*, 1996), and many virulence genes in *Salmonella enterica* serovar Typhimurium (Kelly *et al.*, 2004), *Shigella flexneri* (Falconi *et al.*, 2001), and pathogenic *E. coli* (Goldberg *et al.*, 2001; Sheikh *et al.*, 2001).

IHF is a heterodimeric protein that is approximately 20.5 kDa in size. Unlike the other major nucleoid-associated proteins, IHF binds with relatively high sequence-specificity (Goodrich *et al.*, 1990). IHF is important in controlling DNA architecture, inducing a bend of >160° in bound DNA (Rice *et al.*, 1996). IHF is known to be an important factor in many aspects of DNA metabolism, including replication, recombination, and transcriptional regulation (Friedman, 1988). IHF levels appear to peak during the exponential phase-stationary phase transition, reaching approximately 55,000 monomers per cell, and then decrease to half the cellular maximum in stationary phase (Ali Azam *et al.*, 1999). It is also known to be a critical component of the F plasmid relaxosome, a nucleosomal complex responsible for nicking and unwinding plasmid DNA during transfer (Howard *et al.*, 1995). Studies have also suggested a role in F plasmid transfer gene expression, although the exact mechanism remains unclear, as conflicting studies have suggested both positive and negative regulatory roles (Gamas *et al.*, 1987; Silverman *et al.*, 1991a).

HU is a small, basic, dimeric protein composed of subunits approximately 9.5 kDa in size. Like IHF, these subunits are encoded by two separate but related genes, *hupA* (Kano *et al.*, 1985), and *hupB* (Kano *et al.*, 1986) which encode the α and β subunits, respectively. However, unlike IHF, HU can form both α2 homodimers, which are predominant in exponential phase, and αβ heterodimers, which are predominant in

stationary phase (Claret and Rouviere-Yaniv, 1997). Overall, protein levels follow a profile similar to IHF levels, peaking during the exponential-stationary phase transition at approximately 30,000 dimers, and then decreasing to approximately one third of the cellular maximum during stationary phase (Ali Azam *et al.*, 1999). HU binds DNA in a structure-specific manner, recognizing kinked, cruciform (Pontiggia *et al.*, 1993; Bonnefoy *et al.*, 1994) or nicked DNA (Castaing *et al.*, 1995). Like other bacterial histone-like proteins, it has been shown to be active in all aspects of DNA metabolism, including replication (Dixon and Kornberg, 1984; Bramhill and Kornberg, 1988), recombination (Craigie *et al.*, 1985; Johnson *et al.*, 1986; Morisato and Kleckner, 1987), and transcriptional regulation (Aki *et al.*, 1996).

StpA is an H-NS paralogue, sharing 58% identity with H-NS at the amino acid level (Zhang and Belfort, 1992). Originally characterized as a suppressor of defective *td* intron splicing in the T4 bacteriophage (Zhang and Belfort, 1992; Zhang *et al.*, 1995), StpA shares many properties with H-NS. StpA is capable of binding intrinsically curved DNA and repressing transcription (Zhang *et al.*, 1996), much like H-NS, even complementing *hns* mutations when overexpressed in some cases (Shi and Bennett, 1994). However, unlike H-NS, StpA also appears to act as an RNA chaperone, as described for the *td* intron (Zhang *et al.*, 1995). *stpA* is strongly repressed by H-NS, and is transcribed only transiently during exponential phase (Free and Dorman, 1997). However, *stpA* transcription is activated by growth in minimal media, or in response to environmental stimuli such as osmotic shock and temperature shift (Free and Dorman, 1997). Similar to HU, StpA is capable of forming heteromeric complexes with H-NS and

related proteins, including Sfh, another H-NS paralogue in *Shigella flexneri* (Beloin *et al.*, 2003), however the biological function of these heteromers remains unclear.

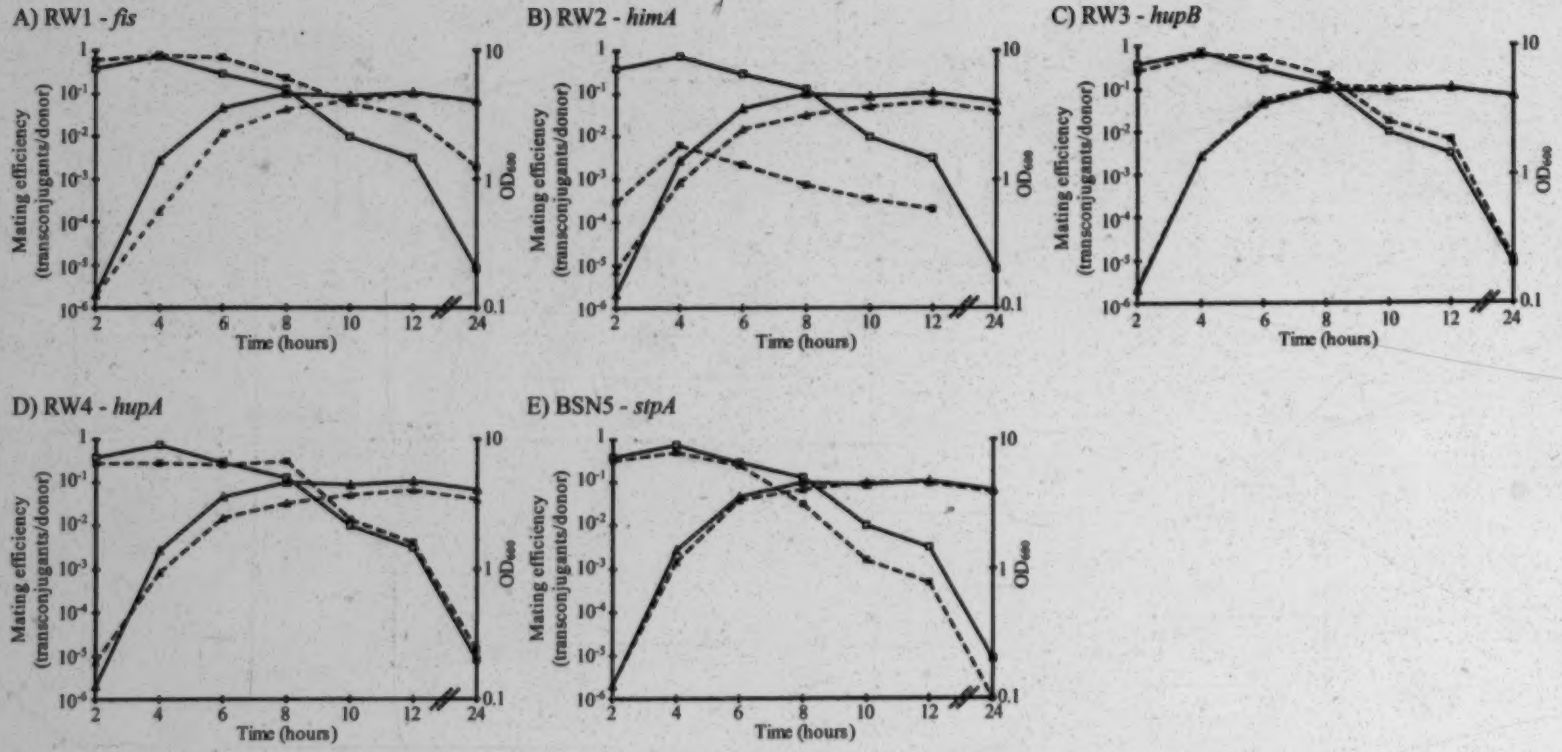
In this study, we examined the biological effects of Fis, HUα, HUβ, IHF, and StpA on F plasmid transfer gene regulation. Our results suggested that two of these factors had detectable effects on plasmid transfer. Fis has a minor negative effect, suggesting a possible role as a repressor. However, IHF has a positive effect on all three regulatory genes: *traJ*, *traM*, and *traY*, suggesting a possible role as an antagonist of H-NS-mediated silencing.

6.2 Results

6.2.1 Mating efficiency analysis of nucleoid-associated protein mutants

To observe the biological effect, if any, of the various nucleoid-associated proteins on F plasmid transfer, the transfer efficiency of pOX38, an F plasmid derivative containing the entire transfer region, was assayed from donor strains containing mutations in the genes encoding each of the host factors. Mating efficiency was assayed at regular intervals throughout growth, as the donor cultures were grown to stationary phase. This was done as regulatory effects on F plasmid transfer are generally most apparent during the transition between late exponential phase and early stationary phase, when transfer efficiency begins to decrease. This was also done because the intracellular levels of nucleoid-associated proteins are generally very dynamic throughout the growth cycle (Ali Azam *et al.*, 1999). Transfer efficiency from the *fis* host strain was prolonged as donor cultures entered stationary phase, and after 24 hours of growth, was 100-fold higher than in the isogenic wild-type host strain, MC4100 (Fig. 6.1A). This suggests that Fis has a negative regulatory role in controlling F plasmid transfer. Transfer efficiency

Figure 6.1 The effect of nucleoid-associated proteins on F plasmid transfer. To determine if any of the major nucleoid-associated proteins had a significant effect on plasmid transfer, the mating efficiencies of donor strains carrying mutations in *fis* (A), *himA* (B), *hupB* (C), *hupA* (D), and *stpA* (E), were tested throughout the growth cycle. At the indicated time-points, samples were removed from donor cell cultures and mated with ED24 to assay mating efficiency (transconjugants/donor). Data for the wild-type strain, MC4100, is indicated by a solid line, whereas data for the mutant strains are indicated by a dashed line. Mating efficiency is indicated by boxes, whereas OD_{600} is indicated by triangles.

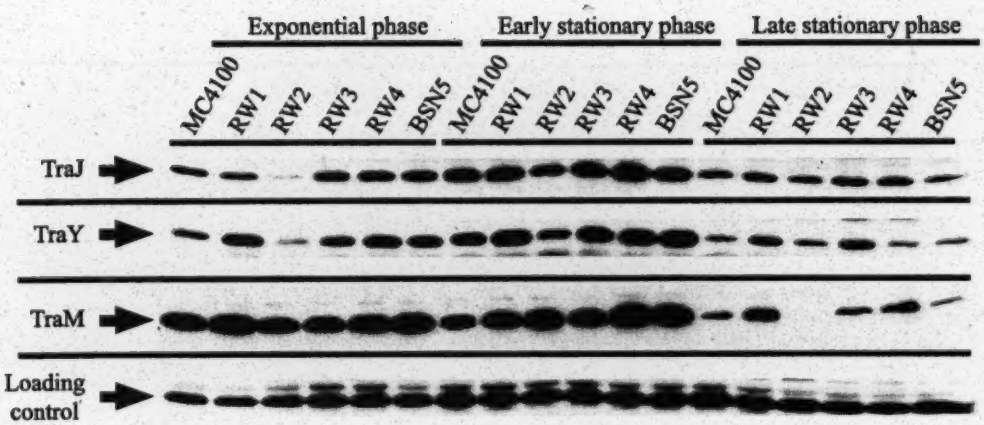

A) RW1 - fis
B) RW2 - himA
C) RW3 - hupB
D) RW4 - hupA
E) BSN5 - stpA
Mating efficiency (transconjugants/donor)
OD600
Time (hours)

from a host strain containing a mutation in *himA*, which encodes one of the IHF subunits, was decreased throughout the growth cycle (Fig. 6.1B). However, plasmid transfer was still readily detectable from the mutant host strain at every time-point except after 24 hours, when mating was no longer detectable. These results are in keeping with previous studies which have demonstrated that IHF is necessary for relaxosome formation (Nelson *et al.*, 1995). No significant change in transfer efficiency was observed for host strains containing mutations in either *hupA* or *hupB*, suggesting that neither of the HU subunits are involved in transfer regulation (Fig. 6.1 C, D). A slight decrease in transfer efficiency from the *stpA* mutant host was observed in stationary phase (Fig. 6.1E). However, the difference between the mutant strain and wild-type was less than an order of magnitude, and was not judged to be significant.

6.2.2 Immunoblot analysis of F plasmid *tra* regulatory proteins in nucleoid-associated protein mutant host strains

To determine if the observed effects on mating efficiency were due to altered expression patterns of the F plasmid regulatory proteins, immunoblot analysis was performed, examining the intracellular levels of TraJ, TraM, and TraY throughout the growth cycle. Cell pellets were collected from cultures in exponential phase at 0.5 OD_{600} (after approximately 3 hours), early stationary phase (8 hours of growth), and late stationary phase (24 hours of growth). Of the five mutant strains tested, the *himA* mutant strain, RW2, was the most strongly affected. TraJ (Fig. 6.2) and TraY levels were clearly decreased in exponential phase donor cultures. TraM levels were also decreased in stationary phase (Fig. 6.2). These results indicate that IHF acts as an activator on

Figure 6.2 The effect of nucleoid-associated proteins on F plasmid Tra regulatory proteins. To assay the effect of the major nucleoid-associated proteins on TraJ, TraY, and TraM levels, immunoblot analysis was performed. Cell pellets were collected from cultures in exponential phase at 0.5 OD_{600} (approximately 3 hours of growth), early stationary phase (8 hours of growth), and late stationary phase (24 hours of growth). A non-specific band was used as a loading control. The genotypes of the experimental strains are as follows: MC4100, wildtype; RW1, *fis*; RW2, *himA*; RW3, *hupB*; RW4, *hupA*; BSN5, *stpA*.

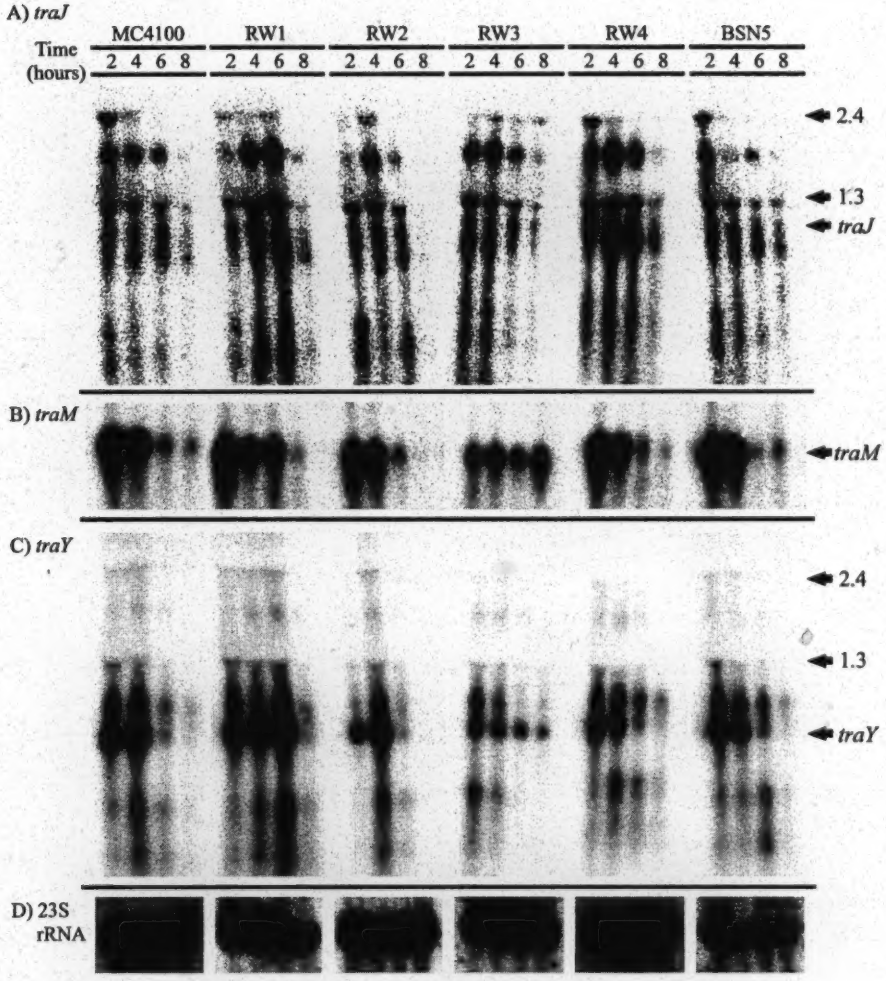

Exponential phase
Early stationary phase
Late stationary phase
MC4100
RW1
RW2
RW3
RW4
BSN5
MC4100
RW1
RW2
RW3
RW4
BSN5
MC4100
RW1
RW2
RW3
RW4
BSN5
TraJ
TraY
TraM
Loading control

tra gene expression. The other mutations had only minor effects on Tra protein levels. Mutation of *fis* in RW1 appeared to cause a slight increase in TraY levels, visible in exponential phase and late stationary phase (Fig. 6.2). The *hupB* mutant strain, RW3, also displayed a slight increase in TraY in late stationary phase, but no other significant effects.

6.2.3 Transcriptional analysis of nucleoid-associated protein mutants

In an attempt to determine if the observed effects on protein levels were due to an effect on transcription, northern blot analysis was performed on donor cultures of each of the mutant host strains. Total cellular RNA was extracted from donor cultures after 2, 4, 6, and 8 hours of growth. As *tra* gene transcription is known to only occur during exponential growth (Will *et al.*, 2004), samples at later time points were not necessary. Each blot was probed with *in vitro* transcribed, [^{32}P]-labelled FinP RNA, which specifically detects *traJ* mRNA (Fig. 6.3A), as well as the [^{32}P]-labelled oligonucleotides SPE5-ext, and RWI78, which detect *traM* (Fig. 6.3B) and *traY* (Fig. 6.3C) transcripts respectively. Again, of the strains tested, the mutation of *himA* in RW2 has the most profound effect on *tra* gene expression. *traJ* and *traY* transcript levels peaked only briefly at 4 hours of growth, as opposed to the more prolonged expression pattern observed in wild-type MC4100. Mutation of *fis* in RW1 appeared to prolong transcription of all three genes, as each transcript is still readily detectable at 6 hours of growth. Mutation of *hupB* also had a minor effect on *traY* and *traM*, resulting in reduced, prolonged transcript levels. Neither *hupA* nor *stpA* had a detectable effect on *tra* gene transcription.

Figure 6.3 F plasmid regulatory transcript levels in nucleoid-associated protein mutant host strains. To determine the effect of *fis*, *himA*, *hupA*, *hupB*, and *stpA* mutations on *traJ* (A), *traM* (B), and *traY* (C) transcript levels, northern blot analysis was performed on total cellular RNA samples collected from each host strain at regular time-points throught growth. Membranes were probed with ^{32}P-labelled FinP RNA, specific to *traJ* (A), ^{32}P-labelled SPE5-ext, specific to *traM* (B), ^{32}P-labelled RWI78, specific to *traY* (C), and ^{32}P-labelled 23SR3, specific to 23S rRNA (D), which was used as a loading control. The host strain and the time of the sample is indicated above the blot. The position of the primary transcript detected by the probe is indicated beside each blot. The genotypes of the experimental strains are as follows: MC4100, wildtype; RW1, *fis*; RW2, *himA*; RW3, *hupB*; RW4, *hupA*; BSN5, *stpA*.


A) traJ
MC4100
RW1
RW2
RW3
RW4
BSN5
Time (hours)
2 4 6 8
2.4
1.3
traJ
B) traM
traM
C) traY
2.4
1.3
traY
D) 23S rRNA

6.3 Discussion

In this study, mutations in genes encoding nucleoid-associated proteins were examined for their effects on F plasmid transfer and *tra* gene expression at various stages of growth. Of the five mutations examined, one, disrupting the *himA* subunit of IHF, had a significant effect, clearly influencing *tra* gene transcription, intracellular protein levels, and transfer efficiency. IHF has long been known to play a role in relaxosome formation, altering local architecture at *oriT* in order to promote nicking and unwinding by the F plasmid relaxase, TraI (Howard *et al.*, 1995). However, the exact role of IHF as a transcriptional regulator has been a point of confusion, as conflicting studies have suggested both positive and negative regulatory roles (Gamas *et al.*, 1987; Dempsey and Fee, 1990; Silverman *et al.*, 1991a; Abo and Ohtsubo, 1993). The data presented here indicate IHF acts as a positive regulator on all three genes, including *traJ* which has not been reported previously.

Based on these results, it seems possible that IHF acts to disrupt H-NS silencing, which represses *tra* gene expression. IHF binding requires major deformation of the duplex, inducing a bend of approximately 160°, and while the IHF consensus binding site is weakly conserved, it contains an A-tract (Goodrich *et al.*, 1990; Rice *et al.*, 1996). As H-NS preferentially binds intrinsically curved, AT-rich DNA (Owen-Hughes *et al.*, 1992), it may compete with IHF for access to potential binding targets. This appears to be the case at *oriT*/P_M where curves 1 and 2 overlap IHF binding sites IHF A and IHF B (Will *et al.*, 2004). However, this does not easily explain activation at the other two promoters. IHF binding sites have not been identified at P_J or P_Y, and while direct transcriptional activation at the promoters is still the most obvious explanation, other

possibilities exist. Increased *traJ* expression may be due to transcriptional read-through from P_M, as numerous read-through transcripts have been observed in previous studies. Alternatively, IHF binding at *oriT* may disrupt a looped repressor complex. Previous studies have demonstrated that *tra* gene regulation is extremely context dependent (Silverman *et al.*, 1991b; Gaudin and Silverman, 1993; Will *et al.*, 2004). Typical H-NS-mediated repression of P_J requires a large segment DNA present *in cis*, including the *oriT* region, which could be due to gene-loop formation between promoters via the strand-bridging activity of H-NS (Will *et al.*, 2004; Dame *et al.*, 2005). Therefore, disruption of H-NS binding at *oriT* could also weaken binding at P_J. However, despite decreased TraM levels in stationary phase, *traM* transcript levels did not appear to change in the *himA* mutant strain, suggesting that IHF may regulate TraM levels indirectly via post-translational mechanisms. Further research is required to determine the mechanism of IHF-mediated activation of P_J and P_Y.

The mutation of *fis* also had a minor effect on F plasmid transfer, prolonging transcription and transfer during entry into stationary phase. However, this is surprising given the dynamic expression profile of *fis*. Intracellular Fis levels peak early in exponential phase, and rapidly decrease to nearly undetectable levels by stationary phase (Ali Azam *et al.*, 1999). The *fis*-dependent effect on plasmid transfer manifests during the transition into stationary phase, suggesting that it is due to indirect effects. One possible explanation is that the effect is due to decreased H-NS levels, as Fis activates expression of *hns* (Falconi *et al.*, 1996). The decreased H-NS levels would result in decreased H-NS-mediated repression of the *tra* promoters. However, it is still possible that Fis directly regulates *tra* gene expression, as Fis has been shown to bind the P_Y

region specifically (see Chapter 10). It is also possible that Fis plays a specialized regulatory role, where its full effect is only apparent under certain growth conditions not examined in this study. The *fis*-dependent effect may also simply be due to a difference in growth rates between the wild-type strain and the slower growing *fis* mutant strain. Mutation of *hupB* appears to have a slight effect on transcript levels, but it does not appear to be significant enough to cause a subsequent change in mating efficiency, and again may be due to indirect effects.

Nucleoid-associated proteins are well-suited to regulating horizontally-acquired genes due to their relative lack of binding specificity. As a result, they are capable of binding a wide array of targets. This property allows these proteins to regulate newly acquired genes that might be "blind" to more specific regulatory signals. This study demonstrates that IHF acts as a positive regulator for expression of all three F plasmid regulatory *tra* genes.

Chapter 7: General Discussion

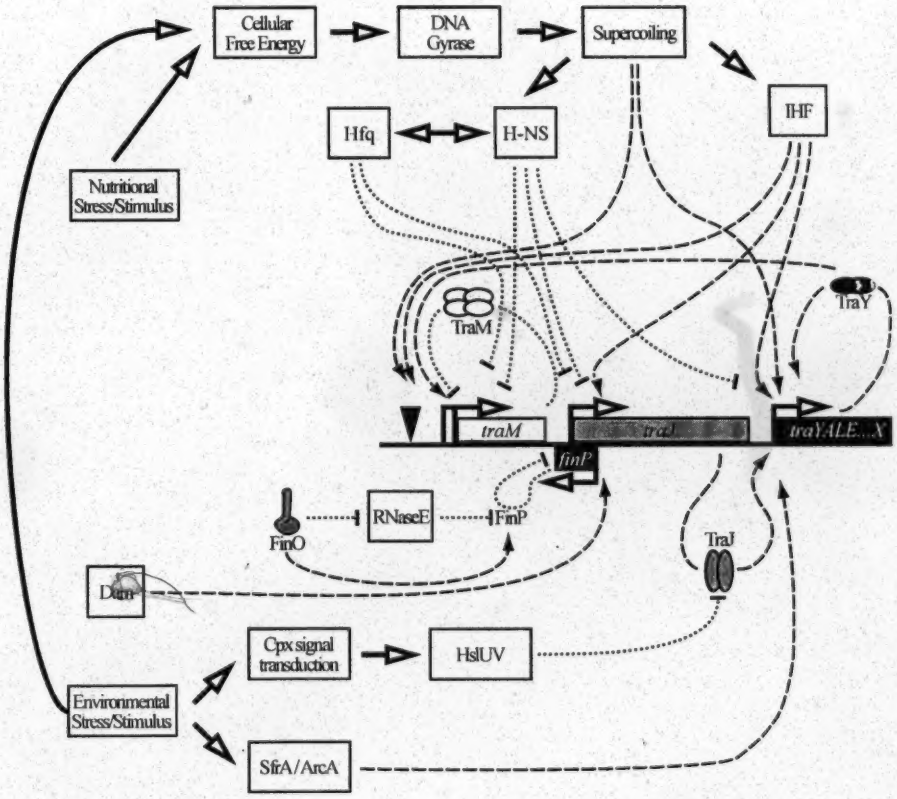
7.1 H-NS: the master repressor of *tra* gene expression

F plasmid *tra* gene expression and plasmid transfer are dynamic processes. Both mating efficiency and *tra* gene transcription peak in exponential phase, but decrease to nearly undetectable levels in stationary phase (Frost and Manchak, 1998; Will *et al.*, 2004). However, until this study, the reason for this was unknown. The results presented in Chapters 3 and 4 of this thesis indicate that H-NS is the central repressor for expression of all F plasmid *tra* genes (Fig. 7.1). *In vitro* binding studies demonstrate that H-NS binds to regions of predicted intrinsic curvature at P_J, P_Y, and P_M. Furthermore, *tra* gene expression was derepressed in *hns* mutant strains, indicating that H-NS acts to shut off *tra* gene expression and mating during the transition into stationary phase. Genetic analysis demonstrated that TraJ, essential for expression of the *tra* operon and mating in wild-type host cells (Willetts, 1977), is not required in an *hns* host, suggesting that TraJ acts to oppose H-NS mediated repression. Similarly, TraY, which is necessary for normal activation of P_M in wild-type host cells (Penfold *et al.*, 1996), is not required for transcription from P_M in an *hns* host. These results suggest that *tra* activators oppose H-NS-mediated repression of their target promoters.

7.2 *tra* silencing is context dependent

Genetic analysis indicated that H-NS-mediated repression of the *tra* system is extremely context dependent, requiring several of the *tra* promoters to be present *in cis* to each other. This may be the result of modulation of H-NS binding due to structural changes in the local duplex due to transcriptionally-generated supercoiling in the *tra* control region, particularly between the convergent P_J and P_{finP} promoters. The circuit could also be affected by transcriptional read-through, particularly from the strong P_M

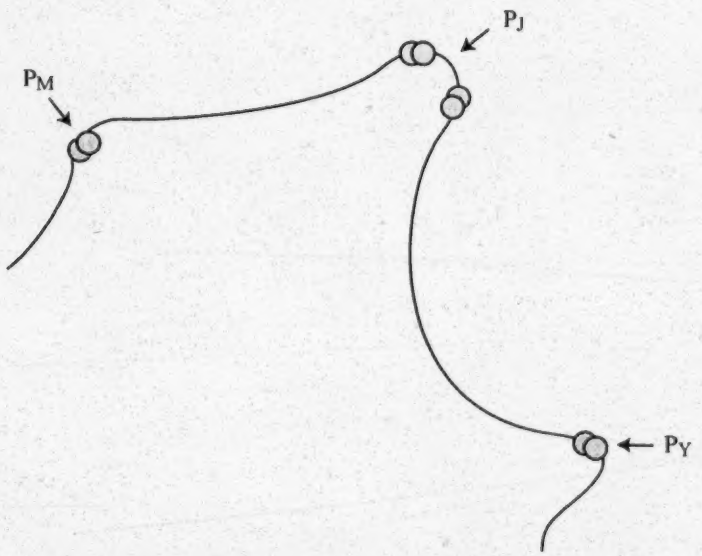
Figure 7.1 Regulation of F plasmid transfer. The F plasmid regulatory circuit consists of three plasmid-encoded regulatory factors: TraJ, TraM, and TraY. The primary activator, TraJ, upregulates the polycistronic *tra* operon, which encodes most of the proteins necessary for transfer, including TraY. TraY further regulates *tra* operon expression and activates transcription of *traM*. TraM is then thought to repress its own expression. However, circuit receives regulatory input from several other factors, which are summarized in this figure. Negative effects are indicated by short dashed lines ending in a bar. Positive effects are indicated by long dashed lines ending in an arrowhead. General host regulatory pathways are indicated by solid, bold lines. It should be noted that regulatory effects reported in F-like plasmids, but have not been confirmed in F, have been omitted.


Cellular Free Energy
DNA Gyrase
Supercoiling
IHF
Hfq
H-NS
Nutritional Stress/Stimulus
TraM
TraY
traM
traJ
traYALE...X
finP
RNaseE
FinP
FinO
TraJ
Dam
Cpx signal transduction
HslUV
Environmental Stress/Stimulus
SfrA/ArcA

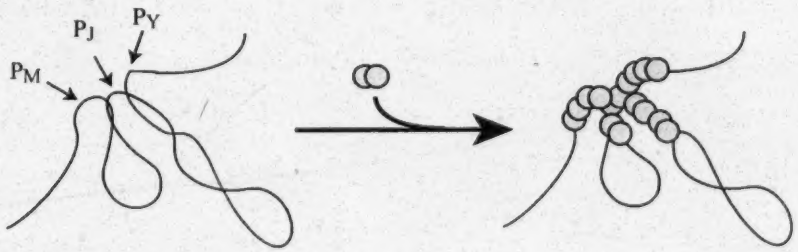
promoter. Alternatively, complete H-NS-mediated repression may involve the formation of gene-loops between H-NS bound at individual promoters. H-NS is known to be capable of bridging two parallel strands, or distant sites on the same strand of DNA (Dame *et al.*, 2005), and this activity is known to be critical in the recognition of intrinsically curved DNA (Dame *et al.*, 2001), but may have additional functions. Recent studies indicate that large gene-loops are common features of eukaryotic gene regulation. The best studied example is activation by upstream enhancer elements at β-globin locus control region (LCR) in mice (Carter *et al.*, 2002; Tolhuis *et al.*, 2002). Gene-loops have also been shown to connect transcriptional initiation and termination by forming between the promoter and terminator of genes in *Saccharomyces cerevisiae*, defining individual transcriptional domains (O'Sullivan *et al.*, 2004). Loop formation between H-NS bound at each *tra* promoter would allow coordinate, cooperative regulation of all of the *tra* genes, as well as limit transcriptional read-through by defining each operon as an individual transcriptional domain. The superhelical energy generated by each of the promoters in the region would cause plectonemic interwinding, which might help to bring the individual promoters into close contact, promoting strand-bridging (Fig. 7.2). The end result might be a single, three-dimensional nucleosomal complex, bound to one-dimensionally distant sites on the DNA. In this case, occlusion or disruption of H-NS binding at one *tra* promoter might simultaneously alter binding at the other *tra* promoters as well. This could explain previous observations that TraM, an autorepressor, is capable of activating *traJ* (Polzleitner *et al.*, 1997), even though no TraM binding sites have been identified near P_J. TraM might bind at P_M and disrupt or weaken H-NS binding at P_M, weakening the nucleosomal complex, and hence, weakening repression at P_J. As a result,

Figure 7.2 A model for three-dimensional nucleation and cooperative silencing. While H-NS (grey circles) may only exhibit limited specific binding at regions containing intrinsic curvature, such as the *tra* promoters, P_M, P_J, and P_Y, on relaxed DNA fragments (A), supercoiling and plectonemic interwinding of the DNA duplex may bring regions into close proximity (B). This might allow strand-bridging between the separate binding sites and the formation of a three-dimensional, cooperative silencing complex.

A)



B)



not only would *tra* gene expression be coordinately repressed as a single unit, but it would be extremely sensitive to activating cues, as activation of any one gene would cause weakening of the entire repressor complex.

The observation that TraM and TraY have both negative and positive regulatory effects on *tra* gene expression highlights another important consideration when studying systems subject to H-NS-mediated silencing. Does the effect of other regulatory proteins involved change when the system is in its de-repressed state, unbound by H-NS, when compared to the system in its silenced state, bound by H-NS? A protein which represses gene expression when the *tra* circuit is free of H-NS-mediated repression, may have a net activational effect when H-NS is present, due to competition between the two proteins and disruption of H-NS binding. This "two-state" model adds another level of complexity to H-NS-regulated systems.

7.3 A new role for TraJ: H-NS antagonist

TraJ appears to be the focal point of control for *tra* regulation. It is subject to transcriptional, post-transcriptional, and post-translational control from numerous factors, and is essential for activation of *tra* gene expression. However, despite its role as primary activator of *tra* gene expression, little is known about its mode of action. As a result, the finding that TraJ is no longer required for transfer in an *hns* mutant host is particularly significant. Earlier studies have suggested that TraJ activity is context dependent, suggesting the presence of a higher-order repressor complex (Silverman *et al.*, 1991b), and have equated TraJ activity *in vivo* with a requirement for P_Y to be supercoiled for transcription *in vitro* (Gaudin and Silverman, 1993). Based on these observations, it was suggested that TraJ acted by disrupting an unidentified nucleosomal

complex which altered local supercoiling at P_Y. Unfortunately, little else is known about TraJ. The TraJ proteins of the F plasmid are extremely poorly conserved (Frost *et al.*, 1994), and despite having a predicted helix-loop-helix domain in the C-terminus, F TraJ has never been demonstrated to bind DNA. The only F-like TraJ protein shown to bind DNA is R100 TraJ, which could only bind DNA at an acidic pH, and even then, the binding activity was highly unstable (Taki *et al.*, 1998). The data presented here suggest that H-NS is a critical component of the repressor complex, and that TraJ opposes this complex, possibly by binding local DNA and occluding or disrupting H-NS binding, or by interacting directly with H-NS.

To better understand regulation of *tra* gene expression, it is worth considering the *vir* regulatory system of *S. flexneri*. Like the F plasmid, *vir* gene expression is regulated by an activation cascade, consisting of the proteins VirF and VirB (Dorman and Porter, 1998). VirF, like TraJ, is the primary activator of *vir* expression, and is thought to disrupt H-NS-mediated repression at *virB*, which in turn, induces expression of all downstream virulence genes. However, the mechanism of VirF-mediated regulation is unclear. Attempts at purifying active, soluble VirF for *in vitro* analysis have met with little success (Porter and Dorman, 2002). DNA binding activity has only been reported once, with a supercoiled target (Tobe *et al.*, 1993). The same study also indicated that the *virB* promoter had to be supercoiled for VirF-mediated activation to occur *in vitro*. Based on these results, it was suggested that disruption of H-NS mediated silencing at *virB* by VirF may alter local topology, similar to the model suggested for TraJ. Although VirF, belonging to the AraC family of DNA binding proteins (Porter and Dorman, 1998), bears no homology to TraJ, there are many functional similarities. The apparent requirement

for a supercoiled target is particularly intriguing, given similar requirements for P_Y activity *in vitro* (Gaudin and Silverman, 1993), as it suggests that TraJ DNA binding and activity might also require supercoiling. The functional similarities hint at the basic characteristics of H-NS antagonist proteins, particularly, a dependence on DNA topology for activity.

7.4 IHF activates plasmid transfer on multiple levels

While IHF has long been known to be involved in F plasmid relaxosome formation (Howard *et al.*, 1995; Nelson *et al.*, 1995), data regarding its role in transcriptional regulation of the *tra* genes is limited and often contradictory. The data presented in Chapter 6 of this thesis indicates that IHF activates transcription of *traJ* and *traY*, and up-regulates TraM levels by an unknown mechanism. While previous studies have suggested a possible activational role on *tra* operon expression, the effect appeared to be minor (Silverman *et al.*, 1991a). Other studies have suggested both positive and negative regulatory roles at P_M (Dempsey and Fee, 1990; Abo and Ohtsubo, 1993), however the results here indicate a positive regulatory role. The results in this thesis are also the first report of an activational effect on *traJ*. As the IHF binding sites at *oriT*, IHFA and IHFB, both partially overlap predicted curves, it seems likely that IHF is competing with H-NS for access to binding sites. Similarly, IHF appears to bind a region of DNA overlapping predicted curves upstream of P_Y, where it likely also competes with H-NS (see Chapter 10). Although no IHF binding site has been identified upstream of *traJ*, it may also be occluding H-NS binding there. Alternatively, perhaps binding at *oriT* is sufficient to disrupt any higher-order repressor complex which might be forming

between the promoters, although this model is contingent on higher-order complex formation.

7.5 Hfq stimulates degradation of transcripts containing the *traJ* 5' UTR

The results presented in Chapter 5 of this thesis indicate that Hfq regulates F plasmid transfer by destabilizing transcripts containing the *traJ* 5' UTR, including polycistronic transcripts generated by transcriptional read-through from P_M. This is a relatively novel finding for Hfq, as it typically targets the 5' termini of transcripts. Targeting of 3' termini has been reported, but appears to be relatively rare, and there are currently no other reports of Hfq targeting intergenic sites. Hfq does not appear to be involved in FinOP-mediated repression of TraJ synthesis, and as no other sRNAs have been shown to regulate *tra* gene expression, it seems likely that the mechanism is similar to that for regulation of *hfq* and *ompA* transcript stability. In these systems, Hfq is thought to alter the secondary structure of the 5' UTR, inhibiting ribosome binding and translation (Vytvytska *et al.*, 2000; Vecerek *et al.*, 2005). The ribonucleases responsible for degrading the *tra* transcripts are currently unknown, however, given the sequence similarity between Hfq binding sites and RNase E cleavage sites, it seems likely that RNase E is involved.

Given the reports of direct interactions between H-NS and Hfq in the regulation of *rpoS*, and the observation that Hfq co-purifies with H-NS (Muffler *et al.*, 1996a), it is interesting to note that the Hfq binding site is also an H-NS binding site (H-NS 4) at the DNA level. Bound H-NS would be in close proximity to the Hfq binding site on nascent transcripts, and might help recruit Hfq to the binding site. Alternatively, increased H-NS binding around P_J might alter the folding kinetics and the secondary structure of the

nascent *traJ* transcript by slowing or stalling the RNA polymerase as it attempts to transcribe *traJ*, altering the structure of the Hfq binding site. Co-regulation by both H-NS and Hfq is likely more widespread than currently documented, as Hfq binding sites often contain H-NS binding sites at the DNA level. AU-rich RNA is the product of AT-rich DNA, which is often intrinsically curved. The required flanking secondary structure is not rare either, as inverted repeats are common elements of many promoters. Reports that H-NS can bind both *rpoS* and DsrA RNA (Brescia *et al.*, 2004), both Hfq targets, also gives weight to the possibility of a significant global role for interactions between these two proteins. Recent studies in *S. cerevisiae* and other eukaryotic organisms have demonstrated many interactions between chromatin associated proteins and RNA processing factors, including transcription-induced gene silencing, where short interfering RNA molecules target chromatin modifying factors to specific genes, altering local chromatin structure and gene expression (Noma *et al.*, 2004; Verdel *et al.*, 2004). While this is not occurring in the *tra* control region, it suggests that interactions between bacterial chromatin proteins like H-NS and RNA chaperones like Hfq are possible. Hopefully future research can illuminate the significance of these interactions.

7.6 Re-programming the host: the role of plasmid-encoded homologs

Sequence analysis has identified a number of H-NS homologs on mobile genetic elements, particularly the IncHI conjugative plasmids (Sherburne *et al.*, 2000; Beloin *et al.*, 2003). Partial homologs, exhibiting similarity to either the DNA-binding domain or the oligomerization domain of H-NS are more widespread. DNA-binding domain homologs have been found in IncM (Nieto and Juarez, 1999) and IncN (More *et al.*, 1996), as well as the LEE pathogenicity island, which may have been acquired

horizontally (Bustamante *et al.*, 2001). These proteins may be capable of competing with H-NS for binding site access, both at plasmid and chromosomal promoters. Oligomerization domain homologs are even more common. Hha, the host-encoded protein homologous to the N-terminal oligomerization domain, is known to interact directly with H-NS (Nieto *et al.*, 2002), with homologs are encoded by IncF, (Nieto and Juarez, 1996), IncHI (Sherburne *et al.*, 2000), and IncM plasmids (Nieto and Juarez, 1999). As Hha family proteins are thought to modulate H-NS activity by forming heteromeric complexes, it seems likely that the plasmid homologs act in the same manner, effectively "high-jacking" chromosomal H-NS. These interactions may result in altered H-NS-mediated regulation of both plasmid and chromosomal genes. Similarly, plasmids and other mobile elements might also code sRNAs which are capable of regulating host genes, possibly in conjunction with Hfq. These possibilities suggest that plasmids and other mobile elements may not simply be passive residents within a host cell, but rather, actively re-program host gene regulation to better suit their individual needs.

7.7 Fine-tuning the signal: the role of additional regulatory factors

While H-NS appears to be the central repressor of the *tra* circuit, numerous other host regulatory factors are involved, particularly at P_J. CRP activates *traJ* expression in the F-like plasmid, pRK100 (Starcic *et al.*, 2003), although preliminary results suggest a more complex role in F (Nartey, Will, and Frost, unpublished results). Studies in pRK100 and pSLT, another F-like plasmid, suggest that Lrp activates *traJ* as well, although no such function has been observed in F (Sultani, Will, and Frost, unpublished

results). These differing results are not necessarily contradictory, but rather, a reflection of the pressure on each plasmid to fine-tune its regulatory circuit to its particular niche.

Transcription from P_J in pSLT is also repressed by Dam methylation at two sites in the region, which is suggested to inhibit binding by Lrp (Camacho and Casadesus, 2005). P_Y is also activated by ArcA, the response regulator of the Arc two-component signal transduction (TCST) system, although the activating signal remains unknown (Strohmaier *et al.*, 1998). While regulation by this many factors may seem unnecessary, it is likely to allow fine-tuning of *tra* gene expression against the overwhelmingly negative signal of H-NS. As H-NS levels are relatively constitutive, there must be a host activational signal to disrupt H-NS silencing, allowing *traJ* expression and upregulation of the rest of the *tra* expression cascade. None of the host factors discussed above appear to provide this activational signal alone, suggesting that activation by the host may involve multiple factors. Activation may also be due to fluctuations in DNA supercoiling, which is already known to influence P_Y activity (Gaudin and Silverman, 1993). During exponential growth, the increase in supercoiling may influence both curvature and the ability of H-NS to bridge specific segments of DNA, hence limiting nucleation and silencing. When supercoiling decreases during stationary phase, the *tra* control region may adopt a topology which better supports nucleation and silencing.

7.8 Evolution of a plasmid regulatory circuit

At first glance, regulation of the F plasmid appears to be extremely complex, perhaps even overly elaborate, subject to multiple signals from both the plasmid and the host. Why this level of redundancy is required is not immediately apparent, but likely occurs for a number of reasons. Initially, selective pressure drives a conjugative plasmid

to evolve in such a way as to ensure maximum plasmid spread throughout a population of suitable recipients. However, for a conjugative plasmid to survive, transfer must also occur when recipient is capable of supporting plasmid establishment and maintenance. This is further complicated if the plasmid carries advantageous traits such as alternative metabolic pathways or virulence genes, as selective pressure is going to drive transfer to occur when these traits are most advantageous to the host cell. While the F plasmid does not appear to carry any of these elements, it has been suggested that it is a genetic "scratchpad" (Gubbins *et al.*, in press), promoting horizontal transfer of host DNA through the formation of co-integrates. If this is the case, transfer is likely most advantageous to the cell during active growth, when replication of the host chromosome is occurring, facilitating recombination. In contrast, the F-like virulence plasmid, pSLT, has evolved a transfer regulatory circuit which is not growth phase-dependent, but promotes transfer under conditions associated with virulence gene expression (Camacho and Casadesus, 2005). However, strong, unregulated gene expression will impose a significant energy cost upon the host cell, and while the genes on a plasmid alone may not seem significant, if they have evolved for high levels of expression, they are likely similar to a much larger group of genes on the host chromosome. The host has evolved in such a way that the same structural elements that support high levels of expression during active growth are targeted by repressors during periods of stress. AT-rich promoters with correctly positioned curvature can promote transcriptional initiation and high levels of expression during oprimal growth conditions, but they can also be targeted by repressors like H-NS. Transfer gene expression evolves to accommodate not just

transfer from the donor cell, but maintenance in the recipient, and expression of other traits carried by the plasmid.

7.9 Future directions

Clearly, a complete mapping of the silencing domain and characterization of the mechanism of H-NS-mediated repression is necessary for future study of *tra* regulation. Any analysis of *tra* transcription without consideration of H-NS will likely be flawed and incomplete. While this study has identified general regions of H-NS binding at the *tra* promoters, a detailed biochemical analysis of H-NS binding throughout the *tra* control region is necessary. This will identify the specific nucleation sites within the *tra* control region, and identify any additional binding sites present within the *tra* coding regions, as curvature prediction analysis suggests the existence of additional H-NS binding sites within *traM* and *traJ* (Will and Frost, unpublished results), which were not studied here. Such a biochemical study should pay special attention to the effect of local topology and supercoiling on H-NS binding, as previous studies have indicated that supercoiling is an important factor in *tra* gene expression (Gaudin and Silverman, 1993). This analysis would also be significant to the study of H-NS, as little is known about the effect of supercoiling on H-NS binding. Structural studies should also be performed to determine if H-NS is promoting loop formation.

After the sites of preferential H-NS binding have been identified, competitive binding studies should be performed to characterize potential interactions between H-NS and other *tra* regulators which might be acting as H-NS antagonists. Again, special consideration should be given to both topology and context, which appear to be important in this system. Typical binding studies utilize small, linear DNA fragments as targets,

however this study may require the use of larger supercoiled targets, as well as *in vivo* studies to observe the true interactions occurring in the *tra* control region. Other factors such as DNA methylation should also be examined. Dam methylation is known to occur at two sites in the P_J/P_{finP} region, where it is thought to inhibit binding of Lrp (Camacho and Casadesus, 2002, 2005). However, the data suggesting that Dam-mediated regulation occurs primarily due to altered Lrp binding is not convincing, and could also be explained by changes in curvature, and hence H-NS binding, due to methylation.

Future research should also examine the role of Hha/YmoA family proteins. A number of plasmids encode their own Hha/YmoA family proteins, including the F-like plasmid, R100 (Nieto and Juarez, 1996). In the case of R100, RmoA, the Hha/YmoA family protein, is known to activate transcription from P_Y (Nieto *et al.*, 1998), although the mechanism is unknown. However, in light of the data presented in this thesis, it seems likely that RmoA interacts with H-NS at P_Y. While there is no RmoA homolog in the F plasmid, the chromosomally-encoded Hha protein or its paralogue, YdgT (Paytubi *et al.*, 2004), may act in a similar capacity.

Studies are also required to determine the mechanism of Hfq-mediated regulation of *tra* gene expression. If Hfq is acting as an RNA chaperone, as with *ompA* and *hfq* (Vytvytska *et al.*, 2000; Moll *et al.*, 2003b; Vecerek *et al.*, 2005), Hfq-dependent structural changes should be detectable *in vitro*. These structural changes should cause a decrease in ribosome binding and translation, which can also be assayed by *in vitro* translation reactions and toe-printing assays. Although *in vitro* studies suggest that sRNAs are not necessary for Hfq-stimulated refolding of *ompA* mRNA, or inhibition of ribosome binding, sRNAs may be involved in the regulation of *traJ* 5'UTR stability.

Chromosomal sRNAs should be analyzed for complementarity to any portion of the *traJ* 5' UTR, to identify any that might be involved in the regulation of TraJ synthesis. The F plasmid should also be screened for putative sRNAs which might be involved.

Care must be taken to consider some of the more novel models for these proteins as well. While H-NS clearly binds DNA at each of the *tra* promoters, the possibility that it also acts post-transcriptionally remains. H-NS might be binding to one of the *tra* transcripts, particularly the *traJ* 5' UTR, which bears some structural similarity to DsrA, a known RNA target of H-NS (Brescia *et al.*, 2004). Perhaps this occurs in conjunction with Hfq activity at the same site. Studies should be undertaken to determine if H-NS is regulating *tra* gene expression post-transcriptionally. Genetic studies should also examine the possibility of cooperative regulation by H-NS and Hfq.

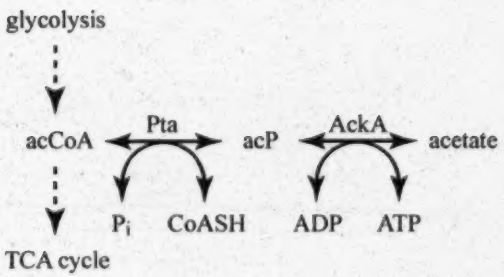
Chapter 8: Appendix I – F plasmid transfer is sensitive to acetate metabolism

8.1 Introduction

Transfer ability of the conjugative F plasmid of *Escherichia coli* is extremely dynamic. Mating efficiency decreases rapidly as donor cell cultures enter stationary phase, and is sensitive to a number of environmental stimuli (Frost and Manchak, 1998; Stallions and Curtiss, 1972). This sensitivity is due to an extremely elaborate regulatory circuit, featuring both host and plasmid-encoded factors. The central regulatory circuit features three plasmid-encoded factors: TraJ, TraY, and TraM. TraJ, encoded by a monocistronic operon, activates expression of the 33 kb polycistronic transfer (*tra*) operon, which encodes most of the proteins necessary for plasmid transfer (Frost *et al.*, 1994). TraY, encoded by the first gene in the *tra* operon, autoregulates its own expression and activates expression of the monocistronic *traM* operon, encoding the autorepressor, *traM*, which is also involved in relaxosome function (Frost *et al.*, 1994). A number of host factors are also involved, including the Arc and Cpx two-component signal transduction (TCST) systems (Silverman *et al.*, 1991a; Silverman *et al.*, 1993). Previous studies suggested that ArcA activates transcription of the *tra* operon in a TraJ-dependent manner (Silverman *et al.*, 1991a; Strohmaier *et al.*, 1998), although the sensor kinase, ArcB, has not been shown to be involved. CpxA/R appears to promote degradation of TraJ, possibly by activating expression of the HslUV protease pair (Gubbins *et al.*, 2002; Lau and Frost, unpublished results).

Acetyl phosphate (acP) is a central intermediate in carbon metabolism, located in the phosphotransacetylase-acetate kinase (Pta-AckA) pathway, which is responsible for the dissimilation of acetate (Fig. 8.1) (Holms, 1996). During periods of aerobic growth when carbon levels exceed the capacity of the tricarboxylic acid (TCA) cycle, excess

Figure 8.1 The Pta-AckA pathway. Phosphotransacetylase (Pta) regenerates coenzyme A (CoASH) from acetyl coenzyme A (acCoA), producing acetyl phosphate (acP). Acetate kinase (AckA) then converts acP to acetate, which is excreted from the cell.


glycolysis
acCoA
Pta
acP
AckA
acetate
P_i
CoASH
ADP
ATP
TCA cycle

acetyl coenzyme-A (acCoA) is directed through the Pta-AckA pathway, generating acetate and ATP, and regenerating coenzyme A, resulting in an increase in acP levels (Brown *et al.*, 1977; Chang *et al.*, 1999). acP levels also increase when oxygen is limited, as acCoA cannot enter the TCA cycle, and must be directed to the Pta-AckA pathway to regenerate CoASH, producing ATP and acetate (el-Mansi and Holms, 1989). As a result, acP levels are generally high under conditions of excess carbon, such as during exponential growth in rich media, or under low oxygen conditions.

acP has garnered attention recently as a potential global regulatory signal, since it was first suggested that acP might act as a phosphoryl donor for *in vivo* autophosphorylation of response regulators in two component signal transduction systems (McCleary *et al.*, 1993; Wanner, 1993; McCleary and Stock, 1994). As at least two TCST response regulators are involved in F plasmid *tra* gene regulation, it seemed possible that F plasmid transfer might be sensitive to intracellular acP pools, particularly in the case of ArcA-mediated activation, for which no cognate sensor kinase has been identified. This study indicates that plasmid transfer and intracellular TraY levels are sensitive to acP and that perturbation of acP synthesis results in a decrease in transfer ability.

8.2 Results and Discussion

8.2.1 Mating efficiency is sensitive to acP accumulation

To observe the effect of acP on F plasmid transfer, host strains containing mutations in either *ackA* or both *ackA* and *pta* in the Pta-AckA pathway were used. *ackA* mutant strains (RW7) can generate acP normally, but are unable to convert it to acetate, and hence, will accumulate acP at a higher rate than wild-type cells. *pta ackA* double

mutants (RW10) are unable to generate acP from the regeneration of acCoA, and hence, will have lower intracellular acP levels than wild-type cells. Donor cultures of RW7, RW10, and the isogenic wild-type strain, JC3272, containing the F plasmid derivative, pOX38, were grown in LB broth to approximately 0.5 OD_{600} and assayed for mating efficiency. RW7 displays a significant decrease in mating efficiency, dropping to approximately 3% of the rate of transfer observed in wild-type donor cells (Table 8.1). However, mating is partially restored in RW10, occurring at 26% of the wild-type rate of transfer. This suggests that accumulation of acP has a negative effect on F plasmid transfer.

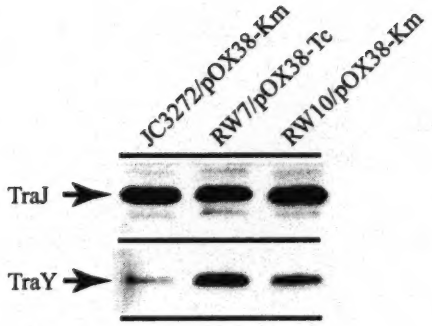
8.2.2 TraY levels are sensitive to acP accumulation

As TCSTs have been suggested to regulate synthesis of both TraJ and TraY, the intracellular levels of these proteins were assayed in JC3272, RW7, and RW10. Cell pellets were collected from exponential phase donor cultures grown in LB broth and assayed for TraJ and TraY using immunoblot analysis (Fig. 8.2). Interestingly, TraJ levels appeared to be unchanged in both RW7 and RW10, despite the fact that both *in vitro* and *in vivo* data from previous studies suggest that CpxR is sensitive to acP (Danese *et al.*, 1995; Danese and Silhavy, 1998). If acP is activating CpxR under the experimental conditions and the current model for Cpx-activated degradation of TraJ is correct, then TraJ should decrease in RW7 donor cultures. However, it is possible that the phosphatase capacity of CpxA can compensate for the increased phosphorylation of CpxR by acP under these conditions, as has been observed in other systems, and a mutation disrupting the kinase, and its corresponding phosphatase activity, may be required to observe any acP-dependent effects on a system (Danese and Silhavy, 1998;

Table 8.1 F plasmid mating efficiency is sensitive to acP accumulation.

Donor strain	Mating efficiency (transconjugants/donor)
JC3272/pOX38-Tc	1.32×10^{-1}
RW7/pOX38-Tc	3.97×10^{-3}
RW10/pOX38-Km	3.46×10^{-2}

Figure 8.2 TraY synthesis is sensitive to acP accumulation. Cell pellets were collected from JC3272, RW7, and RW10 donor cell cultures grown to exponential phase in LB broth and assayed for TraJ and TraY protein levels via immunoblot analysis as indicated.


JC3272/pOX38-Km
RW7/pOX38-Tc
RW10/pOX38-Km
TraJ
TraY

Wolfe, 2005). However, TraY levels were clearly increased in RW7, suggesting that acP activates TraY synthesis. Disruption of the Pta-AckA pathway in RW10, preventing acP accumulation, resulted in near wild-type TraY levels. Based on this, it is possible that acP is allowing autophosphorylation of ArcA, which is then activating transcription of *traY*. What is significant is that observing this acP-dependent event does not require the mutation of a sensor kinase, indicating that acP plays an important role in F plasmid transfer gene regulation. It is also significant in that if this is occurring via ArcA, this case represents the first reported case of acP-mediated autophosphorylation of ArcA *in vivo*.

One issue presented by these results is the role of TraY in regulating F plasmid transfer. Previous studies have suggested that TraY can both positively and negatively regulate *tra* operon expression (Silverman and Sholl, 1996; Taki *et al.*, 1998), but there is no evidence to date of increased TraY levels directly repressing mating. However, as acP accumulation results in increased TraY levels and decreased mating efficiency, it is possible that increased TraY levels might be repressing plasmid transfer, potentially by forming an alternate repressor complex at *oriT*. Alternatively, an as yet unidentified TCST response regulator, or another acP-responsive element, may be repressing transfer by an as yet unidentified mechanism.

Chapter 9: Appendix II – TraJ is post-translationally modified

9.1 Introduction

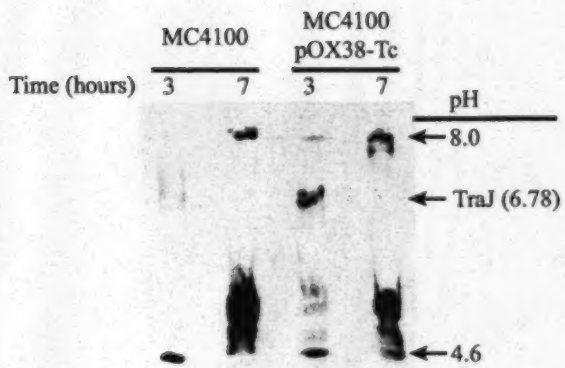
The TraJ protein of the F plasmid is essential for *tra* gene expression and plasmid transfer (Willetts, 1977). TraJ appears to activate transcription of the 33 kb *tra* operon, which encodes most of the proteins necessary for transfer, by opposing H-NS mediated repression at its promoter, P_Y (see Chapter 4). However, the details of TraJ activity remain unclear. F-like TraJ proteins are remarkably dissimilar, particularly when compared to the homology observed in the other F-like plasmid regulators, TraY and TraM (Frost *et al.*, 1994). The TraJ proteins exhibit the greatest homology in the C-terminal domain, which contains a putative helix-loop-helix DNA binding domain (Frost *et al.*, 1994). However, despite the presence of this putative DNA binding domain, F TraJ has not yet been demonstrated to bind DNA. Of all the F-like TraJ proteins, DNA binding activity has only been reported once, for R100, where binding activity was extremely unstable and required acidic binding conditions (Taki *et al.*, 1998). One possible explanation for this is that the DNA binding activity of TraJ either requires or is modulated by some form of post-translational modification. The C-terminal domain of F plasmid TraJ is particularly rich in tyrosine, with 8 tyrosyl residues in the last 72 amino acids, and as a result, phosphorylation of a tyrosyl residue near the putative DNA binding domain could be required for TraJ activity.

9.2 Results and Discussion

To examine the possibility that TraJ might be post-translationally modified isoelectric focusing gel analysis was performed on MC4100/pOX38-Tc lysates. Expression of the *tra* operon is dynamic, peaking in exponential phase and decreasing to undetectable levels in stationary phase (see Chapter 4), which may be reflective of

dynamic modification and activity of TraJ, and in consideration of this, lysates were collected in exponential phase (3 hours of growth), and early stationary phase (7 hours of growth). Samples were then electrophoresed in an isoelectric focusing gel, and examined by immunoblot analysis with α-TraJ antiserum (Fig. 9.1). TraJ has a predicted pI of 6.78 (Frost *et al.*, 1994), and a band is clearly detectable at this pH in the MC4100/pOX38-Tc lysate after 3 hours of growth. There is also a group of faint bands in the acidic range of the gel, suggesting that multiple isoforms of TraJ exist. However, after 7 hours of growth, there is no visible band at pH 6.78, despite the fact that TraJ is detectable throughout the growth cycle when detected using standard SDS-PAGE and immunoblotting procedures (see Chapter 3). This suggests that post-translational modification of TraJ is resulting in a change in pI, and possibly, a loss of activity, as a band at the predicted pH of 6.78 is only detectable during exponential growth, when the *tra* operon is actively transcribed. The acidic species observed after 3 hours are obscured by a large smear after 7 hours, and cannot be examined. This modification does not appear to promote proteolysis as TraJ is detectable throughout growth. Instead, it appears to inhibit the activity of TraJ. As protein phosphorylation is generally associated with transcriptional activation, not repression, other forms of modification may be occurring. TraJ may be extremely sensitive to oxidation, which occurs to many proteins during starvation (Dukan and Nystrom, 1998; Ballesteros *et al.*, 2001). However, the exact nature and role of this modification event is unknown, and requires further biochemical analysis.

Figure 9.1 TraJ is post-translationally modified. Cell lysates were collected after 3 hours and 7 hours of growth from cultures of MC4100 and MC4100/pOX38-Tc. Samples were analyzed by electrophoresis in an isoelectric focusing gel and immunoblotted using α-TraJ antiserum. Sample times are indicated above the gel. The pH range, as well as the predicted position of TraJ, is indicated beside the gel.


MC4100
MC4100
pOX38-Tc
Time (hours)
3
7
3
7
pH
8.0
TraJ (6.78)
4.6

Chapter 10: Appendix III – DNA-binding by Fis and IHF at P_Y

10.1 Introduction

The 33 kb polycistronic transfer (*tra*) operon encodes most of the proteins necessary for plasmid transfer. As a result, regulation of transcription of the *tra* operon from its promoter, P_Y, is an important aspect of F plasmid transfer. As the results presented in Chapter 6 of this thesis suggest that the host nucleoid-associated proteins, IHF and Fis, both affect plasmid transfer, it seemed possible that they might be involved in directly regulating plasmid transfer. Previous studies have already suggested a possible role for IHF in P_Y regulation (Silverman *et al.*, 1991a), and both IHF and Fis are global regulatory proteins, affecting the expression of many host genes (Dorman and Deighan, 1993). To examine the possibility that both Fis and IHF directly regulate transcription from P_Y, *in vitro* binding studies were performed to determine if either IHF or Fis bind the P_Y region in a site-specific manner.

10.2 Results and Discussion

To identify possible IHF and Fis binding sites, the P_Y region was analyzed with the GeneTools software package (BioTools) for potential matches to the IHF binding site consensus sequence (Goodrich *et al.*, 1990), and the Fis consensus binding site (Finkel and Johnson, 1992), which was then checked against the Fis binding site consensus logo (Hengen *et al.*, 1997). One putative IHF binding site was identified, centered at the -63 position relative to P_Y (Fig. 10.1). A putative Fis binding site was identified immediately upstream, centered at the -82 position relative to P_Y (Fig. 10.1). Both sites partially overlap the predicted curve, Y2. To determine if either protein is capable of binding this region, electrophoretic mobility shift assays were performed using pure, native Fis and IHF, and a PCR fragment of the region surrounding P_Y. Both proteins demonstrated

specific binding at P_Y (Fig. 10.2), suggesting that they may be acting directly at P_Y. It is possible that one or both proteins interact or compete with H-NS, as both predicted binding sites partially overlap predicted curve Y2. The predicted IHF binding site also overlaps the putative ArcA binding site (Lynch and Lin, 1996; Strohmaier *et al.*, 1998), however it seems unlikely that both proteins compete for binding site access, as both appear to act as positive regulators. The exact role, if any, for IHF and Fis binding in this region requires further research.

Figure 10.1 Predicted IHF and Fis binding sites upstream of P_Y. The P_Y region was examined for possible IHF and Fis binding sites using the GeneTools software package (BioTools). A putative IHF binding site, indicated by a dashed line along the sequence, was identified upstream of P_Y, centered at the -63 position. A putative Fis binding site, indicated by a solid line along the sequence, was identified centered at the -82 position. P_Y is indicated by an arrow. Regions of predicted curvature are indicated by solid arced lines, as described in Chapter 4. The TraY binding site, *sbyB*, and the putative ArcA binding site are indicated by boxes. The *traY* start codon, the *traY* -10 and -35 boxes, and the *traJ* stop codon are indicated in bold text. A local inverted repeat is indicated by inverted half arrows. Sequence is numbered relative to a BglII site at the start of the transfer region (Frost, *et al.*, 1994).

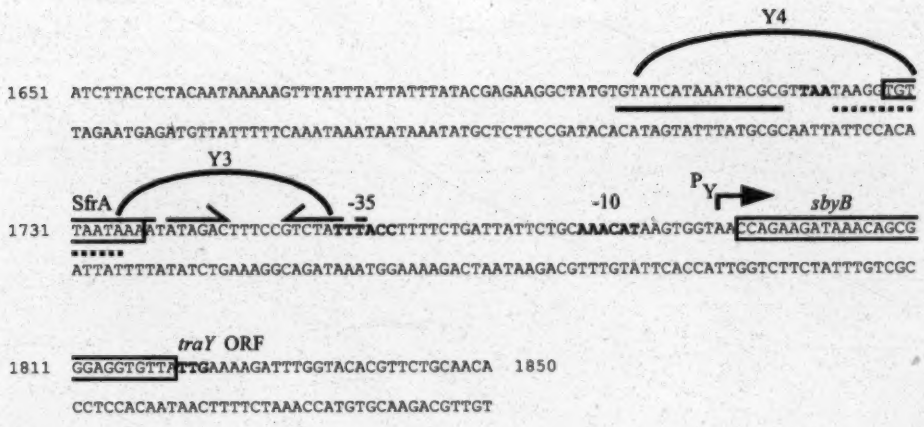
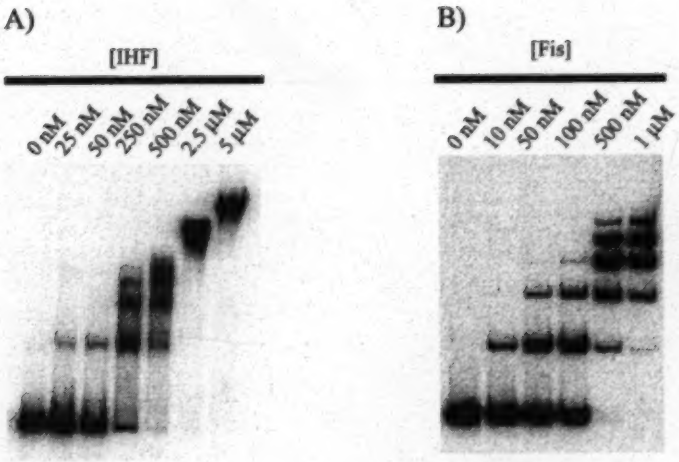

Y4
1651 ATCTTACTCTACAATAAAAAGTTTATTTATTATTTATACGAGAAGGCTATGTGTATCATAAATACGCGTTAATAAGCAGT
TAGAATGAGATGTTATTTTTCAAATAAATAATAAATATGCTCTTCCGATACACATAGTATTTATGCGCAATTATTCCACA
Y3
SfrA
-35
-10
PY
sbyB
1731 TAATAAAATATAGACTTTCCGTCTATTTACCTTTTCTGATTATTCTGCAAACATAAGTGGTAACCAGAAGATAAACAGCG
ATTATTTTATATCTGAAAGGCAGATAAATGGAAAAGACTAATAAGACGTTTGTATTCACCATTGGTCTTCTATTTGTCGC
traY ORF
1811 GGAGGTGTTATTGAAAAGATTTGGTACACGTTCTGCAACA 1850
CCTCCACAATAACTTTTCTAAACCATGTGCAAGACGTTGT

Figure 10.2 IHF and Fis bind the P_Y region. Electrophoretic mobility shift assays were performed to analyze the binding affinity of IHF (A) and Fis (B) for the P_Y region. PCR generated fragment universally labeled with ^{32}P was incubated in the presence of increasing concentrations of protein and electrophoresed in an 8% TBE-polyacrylamide gel. Protein concentrations are indicated above each gel. The relative position of the unbound fragment is indicated beside each gel.


A)
[IHF]
0 nM
25 nM
50 nM
250 nM
500 nM
2.5 μM
5 μM
B)
[Fis]
0 nM
10 nM
50 nM
100 nM
500 nM
1 μM

Chapter 11: References

Abo, T., and Ohtsubo, E. (1993) Repression of the *traM* gene of plasmid R100 by its own product and integration host factor at one of the two promoters. *J Bacteriol* **175**: 4466-4474.

Aboul-ela, F., Bowater, R.P., and Lilley, D.M. (1992) Competing B-Z and helix-coil conformational transitions in supercoiled plasmid DNA. *J Biol Chem* **267**: 1776-1785.

Achtman, M., Willetts, N., and Clark, A.J. (1971) Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterization of transfer-deficient mutants. *J Bacteriol* **106**: 529-538.

Achtman, M., Kennedy, N., and Skurray, R. (1977) Cell--cell interactions in conjugating *Escherichia coli*: role of TraT protein in surface exclusion. *Proc Natl Acad Sci USA* **74**: 5104-5108.

Afflerbach, H., Schroder, O., and Wagner, R. (1998) Effects of the *Escherichia coli* DNA-binding protein H-NS on rRNA synthesis *in vivo*. *Mol Microbiol* **28**: 641-653.

Aki, T., Choy, H.E., and Adhya, S. (1996) Histone-like protein HU as a specific transcriptional regulator: co-factor role in repression of *gal* transcription by GAL repressor. *Genes Cells* **1**: 179-188.

Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999) Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J Bacteriol* **181**: 6361-6370.

Appleman, J.A., Ross, W., Salomon, J., and Gourse, R.L. (1998) Activation of *Escherichia coli* rRNA transcription by FIS during a growth cycle. *J Bacteriol* **180**: 1525-1532.

Arfin, S.M., Long, A.D., Ito, E.T., Tolleri, L., Riehle, M.M., Paegle, E.S., and Hatfield, G.W. (2000) Global gene expression profiling in *Escherichia coli* K12. The effects of integration host factor. *J Biol Chem* **275**: 29672-29684.

Arthur, D.C., Ghetu, A.F., Gubbins, M.J., Edwards, R.A., Frost, L.S., and Glover, J.N. (2003) FinO is an RNA chaperone that facilitates sense-antisense RNA interactions. *EMBO J* **22**: 6346-6355.

Atlung, T., and Hansen, F.G. (2002) Effect of different concentrations of H-NS protein on chromosome replication and the cell cycle in *Escherichia coli*. *J Bacteriol* **184**: 1843-1850.

Azam, T.A., and Ishihama, A. (1999) Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. *J Biol Chem* **274**: 33105-33113.

Azam, T.A., Hiraga, S., and Ishihama, A. (2000) Two types of localization of the DNA-binding proteins within the *Escherichia coli* nucleoid. *Genes Cells* **5**: 613-626.

Badaut, C., Williams, R., Arluison, V., Bouffartigues, E., Robert, B., Buc, H., and Rimsky, S. (2002) The degree of oligomerization of the H-NS nucleoid structuring protein is related to specific binding to DNA. *J Biol Chem* **277**: 41657-41666.

Ball, C.A., and Johnson, R.C. (1991a) Multiple effects of Fis on integration and the control of lysogeny in phage λ. *J Bacteriol* **173**: 4032-4038.

Ball, C.A., and Johnson, R.C. (1991b) Efficient excision of phage λ from the *Escherichia coli* chromosome requires the Fis protein. *J Bacteriol* **173**: 4027-4031.

Ball, C.A., Osuna, R., Ferguson, K.C., and Johnson, R.C. (1992) Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. *J Bacteriol* **174**: 8043-8056.

Ballesteros, M., Fredriksson, A., Henriksson, J., and Nystrom, T. (2001) Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. *EMBO J* **20**: 5280-5289.

Beloin, C., Deighan, P., Doyle, M., and Dorman, C.J. (2003) *Shigella flexneri* 2a strain 2457T expresses three members of the H-NS-like protein family: characterization of the Sfh protein. *Mol Genet Genomics* **270**: 66-77.

Birnboim, H.C., and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**: 1513-1523.

Bloch, V., Yang, Y., Margeat, E., Chavanieu, A., Auge, M.T., Robert, B., Arold, S., Rimsky, S., and Kochoyan, M. (2003) The H-NS dimerization domain defines a new fold contributing to DNA recognition. *Nat Struct Biol* **10**: 212-218.

Bonnefoy, E., Takahashi, M., and Yaniv, J.R. (1994) DNA-binding parameters of the HU protein of *Escherichia coli* to cruciform DNA. *J Mol Biol* **242**: 116-129.

Bramhill, D., and Kornberg, A. (1988) A model for initiation at origins of DNA replication. *Cell* **54**: 915-918.

Brescia, C.C., Mikulecky, P.J., Feig, A.L., and Sledjeski, D.D. (2003) Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure. *RNA* **9**: 33-43.

Brescia, C.C., Kaw, M.K., and Sledjeski, D.D. (2004) The DNA binding protein H-NS binds to and alters the stability of RNA *in vitro* and *in vivo*. *J Mol Biol* **339**: 505-514.

Brown, L., and Elliott, T. (1997) Mutations that increase expression of the *rpoS* gene and decrease its dependence on *hfq* function in *Salmonella typhimurium*. *J Bacteriol* **179**: 656-662.

Brown, T.D., Jones-Mortimer, M.C., and Kornberg, H.L. (1977) The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. *J Gen Microbiol* **102**: 327-336.

Buchrieser, C., Glaser, P., Rusniok, C., Nedjari, H., D'Hauteville, H., Kunst, F., Sansonetti, P., and Parsot, C. (2000) The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Mol Microbiol* **38**: 760-771.

Bustamante, V.H., Santana, F.J., Calva, E., and Puente, J.L. (2001) Transcriptional regulation of type III secretion genes in enteropathogenic *Escherichia coli*: Ler antagonizes H-NS-dependent repression. *Mol Microbiol* **39**: 664-678.

Byrd, D.R., and Matson, S.W. (1997) Nicking by transesterification: the reaction catalysed by a relaxase. *Mol Microbiol* **25**: 1011-1022.

Camacho, E.M., and Casadesus, J. (2002) Conjugal transfer of the virulence plasmid of *Salmonella enterica* is regulated by the leucine-responsive regulatory protein and DNA adenine methylation. *Mol Microbiol* **44**: 1589-1598.

Camacho, E.M., and Casadesus, J. (2005) Regulation of *traJ* transcription in the *Salmonella* virulence plasmid by strand-specific DNA adenine hemimethylation. *Mol Microbiol* **57**: 1700-1718.

Carter, D., Chakalova, L., Osborne, C.S., Dai, Y.F., and Fraser, P. (2002) Long-range chromatin regulatory interactions *in vivo*. *Nat Genet* **32**: 623-626.

Castaing, B., Zelwer, C., Laval, J., and Boiteux, S. (1995) HU protein of *Escherichia coli* binds specifically to DNA that contains single-strand breaks or gaps. *J Biol Chem* **270**: 10291-10296.

Cerdan, R., Bloch, V., Yang, Y., Bertin, P., Dumas, C., Rimsky, S., Kochoyan, M., and Arold, S.T. (2003) Crystal structure of the N-terminal dimerisation domain of VicH, the H-NS-like protein of *Vibrio cholerae*. *J Mol Biol* **334**: 179-185.

Champoux, J.J. (2001) DNA topoisomerases: structure, function, and mechanism. *Annu Rev Biochem* **70**: 369-413.

Chandler, M., and Galas, D.J. (1983) Cointegrate formation mediated by Tn*9*. II. Activity of IS*1* is modulated by external DNA sequences. *J Mol Biol* **170**: 61-91.

Chang, D.E., Shin, S., Rhee, J.S., and Pan, J.G. (1999) Acetate metabolism in a *pta* mutant of *Escherichia coli* W3110: importance of maintaining acetyl coenzyme A flux for growth and survival. *J Bacteriol* **181**: 6656-6663.

Cheah, K.C., and Skurray, R. (1986) The F plasmid carries an IS*3* insertion within *finO*. *J Gen Microbiol* **132**: 3269-3275.

Christie, P.J. (2001) Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol Microbiol* **40**: 294-305.

Claret, L., and Rouviere-Yaniv, J. (1997) Variation in HU composition during growth of *Escherichia coli*: the heterodimer is required for long term survival. *J Mol Biol* **273**: 93-104.

Clewell, D.B., Flannagan, S.E., and Jaworski, D.D. (1995) Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. *Trends Microbiol* **3**: 229-236.

Coulombe, B., and Burton, Z.F. (1999) DNA bending and wrapping around RNA polymerase: a "revolutionary" model describing transcriptional mechanisms. *Microbiol Mol Biol Rev* **63**: 457-478.

Craigie, R., Arndt-Jovin, D.J., and Mizuuchi, K. (1985) A defined system for the DNA strand-transfer reaction at the initiation of bacteriophage Mu transposition: protein and DNA substrate requirements. *Proc Natl Acad Sci USA* **82**: 7570-7574.

Dame, R.T., Wyman, C., and Goosen, N. (2000) H-NS mediated compaction of DNA visualised by atomic force microscopy. *Nucleic Acids Res* **28**: 3504-3510.

Dame, R.T., Wyman, C., and Goosen, N. (2001) Structural basis for preferential binding of H-NS to curved DNA. *Biochimie* **83**: 231-234.

Dame, R.T., Wyman, C., Wurm, R., Wagner, R., and Goosen, N. (2002) Structural basis for H-NS-mediated trapping of RNA polymerase in the open initiation complex at the *rrnB* P1. *J Biol Chem* **277**: 2146-2150.

Dame, R.T., Luijsterburg, M.S., Krin, E., Bertin, P.N., Wagner, R., and Wuite, G.J. (2005) DNA bridging: a property shared among H-NS-like proteins. *J Bacteriol* **187**: 1845-1848.

Danese, P.N., Snyder, W.B., Cosma, C.L., Davis, L.J., and Silhavy, T.J. (1995) The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes Dev* **9**: 387-398.

Danese, P.N., and Silhavy, T.J. (1998) CpxP, a stress-combative member of the Cpx regulon. *J Bacteriol* **180**: 831-839.

Davison, J. (1999) Genetic exchange between bacteria in the environment. *Plasmid* **42**: 73-91.

Dayn, A., Malkhosyan, S., and Mirkin, S.M. (1992) Transcriptionally driven cruciform formation *in vivo*. *Nucleic Acids Res* **20**: 5991-5997.

Dempsey, W.B., and Fee, B.E. (1990) Integration host factor affects expression of two genes at the conjugal transfer origin of plasmid R100. *Mol Microbiol* **4**: 1019-1028.

Disque-Kochem, C., and Dreiseikelmann, B. (1997) The cytoplasmic DNA-binding protein TraM binds to the inner membrane protein TraD *in vitro*. *J Bacteriol* **179**: 6133-6137.

Ditto, M.D., Roberts, D., and Weisberg, R.A. (1994) Growth phase variation of integration host factor level in *Escherichia coli*. *J Bacteriol* **176**: 3738-3748.

Dixon, N.E., and Kornberg, A. (1984) Protein HU in the enzymatic replication of the chromosomal origin of *Escherichia coli*. *Proc Natl Acad Sci USA* **81**: 424-428.

Dole, S., Klingen, Y., Nagarajavel, V., and Schnetz, K. (2004) The protease Lon and the RNA-binding protein Hfq reduce silencing of the *Escherichia coli bgl* operon by H-NS. *J Bacteriol* **186**: 2708-2716.

Donato, G.M., and Kawula, T.H. (1998) Enhanced binding of altered H-NS protein to flagellar rotor protein FliG causes increased flagellar rotational speed and hypermotility in *Escherichia coli*. *J Biol Chem* **273**: 24030-24036.

Dorman, C.J., and Porter, M.E. (1998) The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. *Mol Microbiol* **29**: 677-684.

Dorman, C.J., and Deighan, P. (2003) Regulation of gene expression by histone-like proteins in bacteria. *Curr Opin Genet Dev* **13**: 179-184.

Dorman, C.J. (2004) H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol* **2**: 391-400.

Drew, H.R., Weeks, J.R., and Travers, A.A. (1985) Negative supercoiling induces spontaneous unwinding of a bacterial promoter. *EMBO J* **4**: 1025-1032.

Dri, A.M., Moreau, P.L., and Rouviere-Yaniv, J. (1992) Role of the histone-like proteins OsmZ and HU in homologous recombination. *Gene* **120**: 11-16.

Drlica, K. (1992) Control of bacterial DNA supercoiling. *Mol Microbiol* **6**: 425-433.

Dukan, S., and Nystrom, T. (1998) Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes Dev* **12**: 3431-3441.

Durrenberger, M.B., Villiger, W., and Bachi, T. (1991) Conjugational junctions: morphology of specific contacts in conjugating *Escherichia coli* bacteria. *J Struct Biol* **107**: 146-156.

el-Mansi, E.M., and Holms, W.H. (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. *J Gen Microbiol* **135**: 2875-2883.

Elliott, S.J., Wainwright, L.A., McDaniel, T.K., Jarvis, K.G., Deng, Y.K., Lai, L.C., McNamara, B.P., Donnenberg, M.S., and Kaper, J.B. (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol Microbiol* **28**: 1-4.

Esposito, D., Petrovic, A., Harris, R., Ono, S., Eccleston, J.F., Mbabaali, A., Haq, I., Higgins, C.F., Hinton, J.C., Driscoll, P.C., and Ladbury, J.E. (2002) H-NS oligomerization domain structure reveals the mechanism for high order self-association of the intact protein. *J Mol Biol* **324**: 841-850.

Falconi, M., Brandi, A., La Teana, A., Gualerzi, C.O., and Pon, C.L. (1996) Antagonistic involvement of FIS and H-NS proteins in the transcriptional control of *hns* expression. *Mol Microbiol* **19**: 965-975.

Falconi, M., Prosseda, G., Giangrossi, M., Beghetto, E., and Colonna, B. (2001) Involvement of FIS in the H-NS-mediated regulation of *virF* gene of *Shigella* and enteroinvasive *Escherichia coli*. *Mol Microbiol* **42**: 439-452.

Fekete, R.A., and Frost, L.S. (2002) Characterizing the DNA contacts and cooperative binding of F plasmid TraM to its cognate sites at *oriT*. *J Biol Chem* **277**: 16705-16711.

Filutowicz, M., Ross, W., Wild, J., and Gourse, R.L. (1992) Involvement of Fis protein in replication of the *Escherichia coli* chromosome. *J Bacteriol* **174**: 398-407.

Finkel, S.E., and Johnson, R.C. (1992) The Fis protein: it's not just for DNA inversion anymore. *Mol Microbiol* **6**: 3257-3265.

Finnegan, D., and Willetts, N. (1972) The nature of the transfer inhibitor of several F-like plasmids. *Mol Gen Genet* **119**: 57-66.

Folichon, M., Arluison, V., Pellegrini, O., Huntzinger, E., Regnier, P., and Hajnsdorf, E. (2003) The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* **31**: 7302-7310.

Forns, N., Banos, R.C., Balsalobre, C., Juarez, A., and Madrid, C. (2005) Temperature-dependent conjugative transfer of R27: role of chromosome- and plasmid-encoded Hha and H-NS proteins. *J Bacteriol* **187**: 3950-3959.

Franze de Fernandez, M.T., Eoyang, L., and August, J.T. (1968) Factor fraction required for the synthesis of bacteriophage Qβ-RNA. *Nature* **219**: 588-590.

Free, A., and Dorman, C.J. (1995) Coupling of Escherichia coli *hns* mRNA levels to DNA synthesis by autoregulation: implications for growth phase control. *Mol Microbiol* **18**: 101-113.

Free, A., and Dorman, C.J. (1997) The *Escherichia coli stpA* gene is transiently expressed during growth in rich medium and is induced in minimal medium and by stress conditions. *J Bacteriol* **179**: 909-918.

Friedman, D.I. (1988) Integration host factor: a protein for all reasons. *Cell* **55**: 545-554.

Frost, L.S., Ippen-Ihler, K., and Skurray, R.A. (1994) Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiol Rev* **58**: 162-210.

Frost, L.S., and Manchak, J. (1998) F- phenocopies: characterization of expression of the F transfer region in stationary phase. *Microbiology* **144**: 2579-2587.

Fu, Y.H., Tsai, M.M., Luo, Y.N., and Deonier, R.C. (1991) Deletion analysis of the F plasmid *oriT* locus. *J Bacteriol* **173**: 1012-1020.

Gaffney, D., Skurray, R., and Willetts, N. (1983) Regulation of the F conjugation genes studied by hybridization and *tra-lacZ* fusion. *J Mol Biol* **168**: 103-122.

Gamas, P., Burger, A.C., Churchward, G., Caro, L., Galas, D., and Chandler, M. (1986) Replication of pSC101: effects of mutations in the *E. coli* DNA binding protein IHF. *Mol Gen Genet* **204**: 85-89.

Gamas, P., Caro, L., Galas, D., and Chandler, M. (1987) Expression of F transfer functions depends on the *Escherichia coli* integration host factor. *Mol Gen Genet* **207**: 302-305.

Gao, Q., Luo, Y., and Deonier, R.C. (1994) Initiation and termination of DNA transfer at F plasmid *oriT*. *Mol Microbiol* **11**: 449-458.

Gaudin, H.M., and Silverman, P.M. (1993) Contributions of promoter context and structure to regulated expression of the F plasmid *traY* promoter in *Escherichia coli* K-12. *Mol Microbiol* **8**: 335-342.

Geissmann, T.A., and Touati, D. (2004) Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J* **23**: 396-405.

Gilmour, M.W., Thomson, N.R., Sanders, M., Parkhill, J., and Taylor, D.E. (2004) The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. *Plasmid* **52**: 182-202.

Goldberg, M.D., Johnson, M., Hinton, J.C., and Williams, P.H. (2001) Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*. *Mol Microbiol* **41**: 549-559.

Goodrich, J.A., Schwartz, M.L., and McClure, W.R. (1990) Searching for and predicting the activity of sites for DNA binding proteins: compilation and analysis of the binding sites for *Escherichia coli* integration host factor (IHF). *Nucleic Acids Res* **18**: 4993-5000.

Gottesman, S. (2004) The small RNA regulators of *Escherichia coli*: roles and mechanisms. *Annu Rev Microbiol* **58**: 303-328.

Gowers, D.M., and Halford, S.E. (2003) Protein motion from non-specific to specific DNA by three-dimensional routes aided by supercoiling. *EMBO J* **22**: 1410-1418.

Gubbins, M.J., Lau, I., Will, W.R., Manchak, J.M., Raivio, T.L., and Frost, L.S. (2002) The positive regulator, TraJ, of the *Escherichia coli* F plasmid is unstable in a *cpxA** background. *J Bacteriol* **184**: 5781-5788.

Gubbins, M.J., Arthur, D.C., Ghetu, A.F., Glover, J.N., and Frost, L.S. (2003) Characterizing the structural features of RNA/RNA interactions of the F-plasmid FinOP fertility inhibition system. *J Biol Chem* **278**: 27663-27671.

Gubbins, M.J., Will, W.R., and Frost, L.S. (2005) The F-plasmid, a paradigm for bacterial conjugation, p.151-206. In *The Dynamic Bacterial Genome*. Mullany, P. (ed). Cambridge, UK: Cambridge University Press.

Haffter, P., and Bickle, T.A. (1987) Purification and DNA-binding properties of FIS and Cin, two proteins required for the bacteriophage P1 site-specific recombination system, *cin*. *J Mol Biol* **198**: 579-587.

Hajnsdorf, E., and Regnier, P. (2000) Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci U S A* **97**: 1501-1505.

Hashimoto, M., Imhoff, B., Ali, M.M., and Kow, Y.W. (2003) HU protein of *Escherichia coli* has a role in the repair of closely opposed lesions in DNA. *J Biol Chem* **278**: 28501-28507.

Hengen, P.N., Bartram, S.L., Stewart, L.E., and Schneider, T.D. (1997) Information analysis of Fis binding sites. *Nucleic Acids Res* **25**: 4994-5002.

Hengge-Aronis, R. (1996) Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* **21**: 887-893.

Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G., and Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52**: 569-584.

Hirsch, M., and Elliott, T. (2005) Fis regulates transcriptional induction of RpoS in *Salmonella enterica*. *J Bacteriol* **187**: 1568-1580.

Hirvonen, C.A., Ross, W., Wozniak, C.E., Marasco, E., Anthony, J.R., Aiyar, S.E., Newburn, V.H., and Gourse, R.L. (2001) Contributions of UP elements and the transcription factor FIS to expression from the seven *rrn* P1 promoters in *Escherichia coli*. *J Bacteriol* **183**: 6305-6314.

Holms, H. (1996) Flux analysis and control of the central metabolic pathways in *Escherichia coli*. *FEMS Microbiol Rev* **19**: 85-116.

Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertuy, A., Le Caer, J.P., Danchin, A., and Bertin, P. (2001) Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. *Mol Microbiol* **40**: 20-36.

Howard, M.T., Nelson, W.C., and Matson, S.W. (1995) Stepwise assembly of a relaxosome at the F plasmid origin of transfer. *J Biol Chem* **270**: 28381-28386.

Hsieh, L.S., Rouviere-Yaniv, J., and Drlica, K. (1991) Bacterial DNA supercoiling and [ATP]/[ADP] ratio: changes associated with salt shock. *J Bacteriol* **173**: 3914-3917.

Huang, J., Schlick, T., and Vologodskii, A. (2001) Dynamics of site juxtaposition in supercoiled DNA. *Proc Natl Acad Sci USA* **98**: 968-973.

Huisman, O., Faelen, M., Girard, D., Jaffe, A., Toussaint, A., and Rouviere-Yaniv, J. (1989) Multiple defects in *Escherichia coli* mutants lacking HU protein. *J Bacteriol* **171**: 3704-3712.

Jerome, L.J., and Frost, L.S. (1999) *In vitro* analysis of the interaction between the FinO protein and FinP antisense RNA of F-like conjugative plasmids. *J Biol Chem* **274**: 10356-10362.

Jerome, L.J., van Biesen, T., and Frost, L.S. (1999) Degradation of FinP antisense RNA from F-like plasmids: the RNA-binding protein, FinO, protects FinP from ribonuclease E. *J Mol Biol* **285**: 1457-1473.

Johnson, R.C., and Simon, M.I. (1985) Hin-mediated site-specific recombination requires two 26 bp recombination sites and a 60 bp recombinational enhancer. *Cell* **41**: 781-791.

Johnson, R.C., Bruist, M.F., and Simon, M.I. (1986) Host protein requirements for *in vitro* site-specific DNA inversion. *Cell* **46**: 531-539.

Johnson, R.C., Ball, C.A., Pfeffer, D., and Simon, M.I. (1988) Isolation of the gene encoding the Hin recombinational enhancer binding protein. *Proc Natl Acad Sci USA* **85**: 3484-3488.

Kahmann, R., Rudt, F., Koch, C., and Mertens, G. (1985) G inversion in bacteriophage Mu DNA is stimulated by a site within the invertase gene and a host factor. *Cell* **41**: 771-780.

Kampmann, M. (2005) Facilitated diffusion in chromatin lattices: mechanistic diversity and regulatory potential. *Mol Microbiol* **57**: 889-899.

Kano, Y., Yoshino, S., Wada, M., Yokoyama, K., Nobuhara, M., and Imamoto, F. (1985) Molecular cloning and nucleotide sequence of the HU-1 gene of *Escherichia coli*. *Mol Gen Genet* **201**: 360-362.

Kano, Y., Wada, M., Nagase, T., and Imamoto, F. (1986) Genetic characterization of the gene *hupB* encoding the HU-1 protein of Escherichia coli. *Gene* **45**: 37-44.

Kar, S., and Adhya, S. (2001) Recruitment of HU by piggyback: a special role of GalR in repressosome assembly. *Genes Dev* **15**: 2273-2281.

Katayama, T., Takata, M., and Sekimizu, K. (1996) The nucleoid protein H-NS facilitates chromosome DNA replication in *Escherichia coli dnaA* mutants. *J Bacteriol* **178**: 5790-5792.

Kawula, T.H., and Orndorff, P.E. (1991) Rapid site-specific DNA inversion in *Escherichia coli* mutants lacking the histonelike protein H-NS. *J Bacteriol* **173**: 4116-4123.

Keane, O.M., and Dorman, C.J. (2003) The *gyr* genes of *Salmonella enterica* serovar Typhimurium are repressed by the factor for inversion stimulation, Fis. *Mol Genet Genomics* **270**: 56-65.

Kelly, A., Goldberg, M.D., Carroll, R.K., Danino, V., Hinton, J.C., and Dorman, C.J. (2004) A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. *Microbiology* **150**: 2037-2053.

Kikuchi, A., Flamm, E., and Weisberg, R.A. (1985) An *Escherichia coli* mutant unable to support site-specific recombination of bacteriophage λ. *J Mol Biol* **183**: 129-140.

Komarova, A.V., Tchufistova, L.S., Dreyfus, M., and Boni, I.V. (2005) AU-rich sequences within 5' untranslated leaders enhance translation and stabilize mRNA in *Escherichia coli*. *J Bacteriol* **187**: 1344-1349.

Koraimann, G., and Hogenauer, G. (1989) A stable core region of the *tra* operon mRNA of plasmid R1-19. *Nucleic Acids Res* **17**: 1283-1298.

Koraimann, G., Teferle, K., Markolin, G., Woger, W., and Hogenauer, G. (1996) The FinOP repressor system of plasmid R1: analysis of the antisense RNA control of *traJ* expression and conjugative DNA transfer. *Mol Microbiol* **21**: 811-821.

Kowalski, D., Natale, D.A., and Eddy, M.J. (1988) Stable DNA unwinding, not "breathing," accounts for single-strand-specific nuclease hypersensitivity of specific A+T-rich sequences. *Proc Natl Acad Sci USA* **85**: 9464-9468.

Kumari, S., Beatty, C.M., Browning, D.F., Busby, S.J., Simel, E.J., Hovel-Miner, G., and Wolfe, A.J. (2000) Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* **182**: 4173-4179.

Kusano, S., Ding, Q., Fujita, N., and Ishihama, A. (1996) Promoter selectivity of *Escherichia coli* RNA polymerase E sigma 70 and E sigma 38 holoenzymes. Effect of DNA supercoiling. *J Biol Chem* **271**: 1998-2004.

Lahue, E.E., and Matson, S.W. (1988) *Escherichia coli* DNA helicase I catalyzes a unidirectional and highly processive unwinding reaction. *J Biol Chem* **263**: 3208-3215.

Lahue, E.E., and Matson, S.W. (1990) Purified *Escherichia coli* F-factor TraY protein binds *oriT*. *J Bacteriol* **172**: 1385-1391.

Laundon, C.H., and Griffith, J.D. (1988) Curved helix segments can uniquely orient the topology of supertwisted DNA. *Cell* **52**: 545-549.

Lawley, T.D., Klimke, W.A., Gubbins, M.J., and Frost, L.S. (2003) F factor conjugation is a true type IV secretion system. *FEMS Microbiol Lett* **224**: 1-15.

Lease, R.A., Cusick, M.E., and Belfort, M. (1998) Riboregulation in *Escherichia coli*: DsrA RNA acts by RNA:RNA interactions at multiple loci. *Proc Natl Acad Sci USA* **95**: 12456-12461.

Lease, R.A., and Belfort, M. (2000a) Riboregulation by DsrA RNA: trans-actions for global economy. *Mol Microbiol* **38**: 667-672.

Lease, R.A., and Belfort, M. (2000b) A trans-acting RNA as a control switch in *Escherichia coli*: DsrA modulates function by forming alternative structures. *Proc Natl Acad Sci USA* **97**: 9919-9924.

Lederberg, J., and Tatum, E.L. (1946) Gene recombination in *Escherichia coli*. *Nature* **158**: 558.

Lee, S.H., Frost, L.S., and Paranchych, W. (1992) FinOP repression of the F plasmid involves extension of the half-life of FinP antisense RNA by FinO. *Mol Gen Genet* **235**: 131-139.

Li, S., and Waters, R. (1998) *Escherichia coli* strains lacking protein HU are UV sensitive due to a role for HU in homologous recombination. *J Bacteriol* **180**: 3750-3756.

Lia, G., Bensimon, D., Croquette, V., Allemand, J.F., Dunlap, D., Lewis, D.E., Adhya, S., and Finzi, L. (2003) Supercoiling and denaturation in Gal repressor/heat unstable nucleoid protein (HU)-mediated DNA looping. *Proc Natl Acad Sci USA* **100**: 11373-11377.

Liu, L.F., and Wang, J.C. (1987) Supercoiling of the DNA template during transcription. *Proc Natl Acad Sci USA* **84**: 7024-7027.

Liu, Q., and Richardson, C.C. (1993) Gene 5.5 protein of bacteriophage T7 inhibits the nucleoid protein H-NS of *Escherichia coli*. *Proc Natl Acad Sci USA* **90**: 1761-1765.

Llosa, M., Gomis-Ruth, F.X., Coll, M., and de la Cruz Fd, F. (2002) Bacterial conjugation: a two-step mechanism for DNA transport. *Mol Microbiol* **45**: 1-8.

Lu, J., and Frost, L.S. (2005) Mutations in the C-terminal region of TraM provide evidence for in vivo TraM-TraD interactions during F-plasmid conjugation. *J Bacteriol* **187**: 4767-4773.

Lynch, A.S., and Lin, E.C. (1996) Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. *J Bacteriol* **178**: 6238-6249.

Mackie, G.A. (1991) Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of *Escherichia coli* requires the product of the ams gene *in vivo* and *in vitro*. *J Bacteriol* **173**: 2488-2497.

Mackie, G.A., and Genereaux, J.L. (1993) The role of RNA structure in determining RNase E-dependent cleavage sites in the mRNA for ribosomal protein S20 *in vitro*. *J Mol Biol* **234**: 998-1012.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci USA* **95**: 12462-12467.

Maneewannakul, K., Kathir, P., Endley, S., Moore, D., Manchak, J., Frost, L., and Ippen-Ihler, K. (1996) Construction of derivatives of the F plasmid pOX-*tra715*: characterization of *traY* and *traD* mutants that can be complemented *in trans*. *Mol Microbiol* **22**: 197-205.

Masse, E., Escorcia, F.E., and Gottesman, S. (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev* **17**: 2374-2383.

Matson, S.W., and Morton, B.S. (1991) *Escherichia coli* DNA helicase I catalyzes a site- and strand-specific nicking reaction at the F plasmid *oriT*. *J Biol Chem* **266**: 16232-16237.

Matson, S.W., Nelson, W.C., and Morton, B.S. (1993) Characterization of the reaction product of the *oriT* nicking reaction catalyzed by *Escherichia coli* DNA helicase I. *J Bacteriol* **175**: 2599-2606.

Maurelli, A.T., and Sansonetti, P.J. (1988) Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. *Proc Natl Acad Sci USA* **85**: 2820-2824.

McCleary, W.R., Stock, J.B., and Ninfa, A.J. (1993) Is acetyl phosphate a global signal in *Escherichia coli*? *J Bacteriol* **175**: 2793-2798.

McCleary, W.R., and Stock, J.B. (1994) Acetyl phosphate and the activation of two-component response regulators. *J Biol Chem* **269**: 31567-31572.

McDowall, K.J., Lin-Chao, S., and Cohen, S.N. (1994) A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage. *J Biol Chem* **269**: 10790-10796.

Mikulecky, P.J., Kaw, M.K., Brescia, C.C., Takach, J.C., Sledjeski, D.D., and Feig, A.L. (2004) *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nat Struct Mol Biol* **11**: 1206-1214.

Miller, H.I., and Friedman, D.I. (1980) An *E. coli* gene product required for λ site-specific recombination. *Cell* **20**: 711-719.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratories.

Moitoso de Vargas, L., Kim, S., and Landy, A. (1989) DNA looping generated by DNA bending protein IHF and the two domains of λ integrase. *Science* **244**: 1457-1461.

Mojica, F.J., and Higgins, C.F. (1997) *In vivo* supercoiling of plasmid and chromosomal DNA in an *Escherichia coli hns* mutant. *J Bacteriol* **179**: 3528-3533.

Moll, I., Afonyushkin, T., Vytvytska, O., Kaberdin, V.R., and Blasi, U. (2003a) Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs. *RNA* **9**: 1308-1314.

Moll, I., Leitsch, D., Steinhauser, T., and Blasi, U. (2003b) RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep* **4**: 284-289.

Møller, T., Franch, T., Højrup, P., Keene, D.R., Bachinger, H.P., Brennan, R.G., and Valentin-Hansen, P. (2002) Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell* **9**: 23-30.

More, M.I., Pohlman, R.F., and Winans, S.C. (1996) Genes encoding the pKM101 conjugal mating pore are negatively regulated by the plasmid-encoded KorA and KorB proteins. *J Bacteriol* **178**: 4392-4399.

Morisato, D., and Kleckner, N. (1987) Tn*10* transposition and circle formation in vitro. *Cell* **51**: 101-111.

Muffler, A., Fischer, D., Altuvia, S., Storz, G., and Hengge-Aronis, R. (1996a) The response regulator RssB controls stability of the sigma(S) subunit of RNA polymerase in *Escherichia coli*. *EMBO J* **15**: 1333-1339.

Muffler, A., Fischer, D., and Hengge-Aronis, R. (1996b) The RNA-binding protein HF-I, known as a host factor for phage Qβ RNA replication, is essential for *rpoS* translation in *Escherichia coli*. *Genes Dev* **10**: 1143-1151.

Muskhelishvili, G., and Travers, A. (2003) Transcription factor as a topological homeostat. *Front Biosci* **8**: d279-285.

Nasser, W., Schneider, R., Travers, A., and Muskhelishvili, G. (2001) CRP modulates *fis* transcription by alternate formation of activating and repressing nucleoprotein complexes. *J Biol Chem* **276**: 17878-17886.

Neidhardt, F.C., Bloch, P.L., and Smith, D.F. (1974) Culture medium for enterobacteria. *J Bacteriol* **119**: 736-747.

Nelson, W.C., Morton, B.S., Lahue, E.E., and Matson, S.W. (1993) Characterization of the *Escherichia coli* F factor *traY* gene product and its binding sites. *J Bacteriol* **175**: 2221-2228.

Nelson, W.C., Howard, M.T., Sherman, J.A., and Matson, S.W. (1995) The *traY* gene product and integration host factor stimulate *Escherichia coli* DNA helicase I-catalyzed nicking at the F plasmid *oriT*. *J Biol Chem* **270**: 28374-28380.

Nieto, J.M., Carmona, M., Bolland, S., Jubete, Y., de la Cruz, F., and Juarez, A. (1991) The *hha* gene modulates haemolysin expression in *Escherichia coli*. *Mol Microbiol* **5**: 1285-1293.

Nieto, J.M., and Juarez, A. (1996) A new member of the Hha/YmoA class of bacterial regulators in plasmid R100 of *Escherichia coli*? *Mol Microbiol* **19**: 407.

Nieto, J.M., Prenafeta, A., Miquelay, E., Torrades, S., and Juarez, A. (1998) Sequence, identification, and effect on conjugation of the *rmoA* gene of plasmid R100-1. *FEMS Microbiol Lett* **169**: 59-66.

Nieto, J.M., and Juarez, A. (1999) The putative Orf4 of broad-host-range conjugative plasmid R446 could be related to the H-NS family of bacterial nucleoid-associated proteins. *Plasmid* **41**: 125-127.

Nieto, J.M., Madrid, C., Prenafeta, A., Miquelay, E., Balsalobre, C., Carrascal, M., and Juarez, A. (2000) Expression of the hemolysin operon in *Escherichia coli* is modulated by a nucleoid-protein complex that includes the proteins Hha and H-NS. *Mol Gen Genet* **263**: 349-358.

Nieto, J.M., Madrid, C., Miquelay, E., Parra, J.L., Rodriguez, S., and Juarez, A. (2002) Evidence for direct protein-protein interaction between members of the enterobacterial Hha/YmoA and H-NS families of proteins. *J Bacteriol* **184**: 629-635.

Ninnemann, O., Koch, C., and Kahmann, R. (1992) The *E.coli fis* promoter is subject to stringent control and autoregulation. *EMBO J* **11**: 1075-1083.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004) RITS acts *in cis* to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* **36**: 1174-1180.

Novotny, C.P., and Fives-Taylor, P. (1974) Retraction of F pili. *J Bacteriol* **117**: 1306-1311.

O'Gara J, P., and Dorman, C.J. (2000) Effects of local transcription and H-NS on inversion of the *fim* switch of *Escherichia coli*. *Mol Microbiol* **36**: 457-466.

O'Sullivan, J.M., Tan-Wong, S.M., Morillon, A., Lee, B., Coles, J., Mellor, J., and Proudfoot, N.J. (2004) Gene loops juxtapose promoters and terminators in yeast. *Nat Genet* **36**: 1014-1018.

Owen-Hughes, T.A., Pavitt, G.D., Santos, D.S., Sidebotham, J.M., Hulton, C.S., Hinton, J.C., and Higgins, C.F. (1992) The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. *Cell* **71**: 255-265.

Owens, R.M., Pritchard, G., Skipp, P., Hodey, M., Connell, S.R., Nierhaus, K.H., and O'Connor, C.D. (2004) A dedicated translation factor controls the synthesis of the global regulator Fis. *EMBO J* **23**: 3375-3385.

Parekh, B.S., Sheridan, S.D., and Hatfield, G.W. (1996) Effects of integration host factor and DNA supercoiling on transcription from the *ilvPG* promoter of *Escherichia coli*. *J Biol Chem* **271**: 20258-20264.

Paytubi, S., Madrid, C., Forns, N., Nieto, J.M., Balsalobre, C., Uhlin, B.E., and Juarez, A. (2004) YdgT, the Hha paralogue in *Escherichia coli*, forms heteromeric complexes with H-NS and StpA. *Mol Microbiol* **54**: 251-263.

Penfold, S.S., Simon, J., and Frost, L.S. (1996) Regulation of the expression of the *traM* gene of the F sex factor of *Escherichia coli*. *Mol Microbiol* **20**: 549-558.

Pettijohn, D.E., and Pfenninger, O. (1980) Supercoils in prokaryotic DNA restrained *in vivo*. *Proc Natl Acad Sci USA* **77**: 1331-1335.

Pinson, V., Takahashi, M., and Rouviere-Yaniv, J. (1999) Differential binding of the *Escherichia coli* HU, homodimeric forms and heterodimeric form to linear, gapped and cruciform DNA. *J Mol Biol* **287**: 485-497.

Polzleitner, E., Zechner, E.L., Renner, W., Fratte, R., Jauk, B., Hogenauer, G., and Koraimann, G. (1997) TraM of plasmid R1 controls transfer gene expression as an integrated control element in a complex regulatory network. *Mol Microbiol* **25**: 495-507.

Pon, C.L., Calogero, R.A., and Gualerzi, C.O. (1988) Identification, cloning, nucleotide sequence and chromosomal map location of *hns*, the structural gene for *Escherichia coli* DNA-binding protein H-NS. *Mol Gen Genet* **212**: 199-202.

Pontiggia, A., Negri, A., Beltrame, M., and Bianchi, M.E. (1993) Protein HU binds specifically to kinked DNA. *Mol Microbiol* **7**: 343-350.

Porter, M.E., and Dorman, C.J. (2002) In vivo DNA-binding and oligomerization properties of the *Shigella flexneri* AraC-like transcriptional regulator VirF as identified by random and site-specific mutagenesis. *J Bacteriol* **184**: 531-539.

Pratt, T.S., Steiner, T., Feldman, L.S., Walker, K.A., and Osuna, R. (1997) Deletion analysis of the *fis* promoter region in *Escherichia coli*: antagonistic effects of integration host factor and Fis. *J Bacteriol* **179**: 6367-6377.

Prosseda, G., Falconi, M., Giangrossi, M., Gualerzi, C.O., Micheli, G., and Colonna, B. (2004) The *virF* promoter in *Shigella*: more than just a curved DNA stretch. *Mol Microbiol* **51**: 523-537.

Raivio, T.L., and Silhavy, T.J. (2001) Periplasmic stress and ECF sigma factors. *Annu Rev Microbiol* **55**: 591-624.

Renzoni, D., Esposito, D., Pfuhl, M., Hinton, J.C., Higgins, C.F., Driscoll, P.C., and Ladbury, J.E. (2001) Structural characterization of the N-terminal oligomerization domain of the bacterial chromatin-structuring protein, H-NS. *J Mol Biol* **306**: 1127-1137.

Reygers, U., Wessel, R., Muller, H., and Hoffman-Berling, H. (1991) Endonuclease activity of *Escherichia coli* DNA helicase I directed against the transfer origin of the F factor. *EMBO J* **10**: 2689-2694.

Rice, P.A., Yang, S., Mizuuchi, K., and Nash, H.A. (1996) Crystal structure of an IHF-DNA complex: a protein-induced DNA U-turn. *Cell* **87**: 1295-1306.

Rimsky, S., Zuber, F., Buckle, M., and Buc, H. (2001) A molecular mechanism for the repression of transcription by the H-NS protein. *Mol Microbiol* **42**: 1311-1323.

Rimsky, S. (2004) Structure of the histone-like protein H-NS and its role in regulation and genome superstructure. *Curr Opin Microbiol* **7**: 109-114.

Rippe, K., von Hippel, P.H., and Langowski, J. (1995) Action at a distance: DNA-looping and initiation of transcription. *Trends Biochem Sci* **20**: 500-506.

Robertson, C.A., and Nash, H.A. (1988) Bending of the bacteriophage λ attachment site by *Escherichia coli* integration host factor. *J Biol Chem* **263**: 3554-3557.

Rouviere-Yaniv, J., Yaniv, M., and Germond, J.E. (1979) *E. coli* DNA binding protein HU forms nucleosomelike structure with circular double-stranded DNA. *Cell* **17**: 265-274.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Santos, P.M., Di Bartolo, I., Blatny, J.M., Zennaro, E., and Valla, S. (2001) New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. *FEMS Microbiol Lett* **195**: 91-96.

Sasakawa, C., Kamata, K., Sakai, T., Makino, S., Yamada, M., Okada, N., and Yoshikawa, M. (1988) Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. *J Bacteriol* **170**: 2480-2484.

Schechter, L.M., Jain, S., Akbar, S., and Lee, C.A. (2003) The small nucleoid-binding proteins H-NS, HU, and Fis affect *hilA* expression in *Salmonella enterica* serovar Typhimurium. *Infect Immun* **71**: 5432-5435.

Schneider, D.A., Ross, W., and Gourse, R.L. (2003) Control of rRNA expression in *Escherichia coli. Curr Opin Microbiol* **6**: 151-156.

Schneider, R., Travers, A., Kutateladze, T., and Muskhelishvili, G. (1999) A DNA architectural protein couples cellular physiology and DNA topology in *Escherichia coli. Mol Microbiol* **34**: 953-964.

Schneider, R., Travers, A., and Muskhelishvili, G. (2000) The expression of the *Escherichia coli fis* gene is strongly dependent on the superhelical density of DNA. *Mol Microbiol* **38**: 167-175.

Schneider, R., Lurz, R., Luder, G., Tolksdorf, C., Travers, A., and Muskhelishvili, G. (2001) An architectural role of the *Escherichia coli* chromatin protein FIS in organising DNA. *Nucleic Acids Res* **29**: 5107-5114.

Schroder, O., and Wagner, R. (2000) The bacterial DNA-binding protein H-NS represses ribosomal RNA transcription by trapping RNA polymerase in the initiation complex. *J Mol Biol* **298**: 737-748.

Schumacher, M.A., Pearson, R.F., Moller, T., Valentin-Hansen, P., and Brennan, R.G. (2002) Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J* **21**: 3546-3556.

Sheikh, J., Hicks, S., Dall'Agnol, M., Phillips, A.D., and Nataro, J.P. (2001) Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. *Mol Microbiol* **41**: 983-997.

Sherburne, C.K., Lawley, T.D., Gilmour, M.W., Blattner, F.R., Burland, V., Grotbeck, E., Rose, D.J., and Taylor, D.E. (2000) The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Res* **28**: 2177-2186.

Sheridan, S.D., Benham, C.J., and Hatfield, G.W. (1998) Activation of gene expression by a novel DNA structural transmission mechanism that requires supercoiling-induced DNA duplex destabilization in an upstream activating sequence. *J Biol Chem* **273**: 21298-21308.

Sheridan, S.D., Benham, C.J., and Hatfield, G.W. (1999) Inhibition of DNA supercoiling-dependent transcriptional activation by a distant B-DNA to Z-DNA transition. *J Biol Chem* **274**: 8169-8174.

Sherman, J.A., and Matson, S.W. (1994) *Escherichia coli* DNA helicase I catalyzes a sequence-specific cleavage/ligation reaction at the F plasmid origin of transfer. *J Biol Chem* **269**: 26220-26226.

Shi, X., and Bennett, G.N. (1994) Plasmids bearing *hfq* and the *hns*-like gene *stpA* complement *hns* mutants in modulating arginine decarboxylase gene expression in *Escherichia coli*. *J Bacteriol* **176**: 6769-6775.

Silverman, P.M., Wickersham, E., and Harris, R. (1991a) Regulation of the F plasmid *traY* promoter in *Escherichia coli* by host and plasmid factors. *J Mol Biol* **218**: 119-128.

Silverman, P.M., Wickersham, E., Rainwater, S., and Harris, R. (1991b) Regulation of the F plasmid *traY* promoter in *Escherichia coli* K12 as a function of sequence context. *J Mol Biol* **220**: 271-279.

Silverman, P.M., Tran, L., Harris, R., and Gaudin, H.M. (1993) Accumulation of the F plasmid TraJ protein in *cpx* mutants of *Escherichia coli*. *J Bacteriol* **175**: 921-925.

Silverman, P.M., and Sholl, A. (1996) Effect of *traY* amber mutations on F-plasmid *traY* promoter activity *in vivo*. *J Bacteriol* **178**: 5787-5789.

Skarstad, K., Baker, T.A., and Kornberg, A. (1990) Strand separation required for initiation of replication at the chromosomal origin of *E.coli* is facilitated by a distant RNA--DNA hybrid. *EMBO J* **9**: 2341-2348.

Sledjeski, D., and Gottesman, S. (1995) A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli*. *Proc Natl Acad Sci USA* **92**: 2003-2007.

Sledjeski, D.D., Whitman, C., and Zhang, A. (2001) Hfq is necessary for regulation by the untranslated RNA DsrA. *J Bacteriol* **183**: 1997-2005.

Smyth, C.P., Lundback, T., Renzoni, D., Siligardi, G., Beavil, R., Layton, M., Sidebotham, J.M., Hinton, J.C., Driscoll, P.C., Higgins, C.F., and Ladbury, J.E. (2000) Oligomerization of the chromatin-structuring protein H-NS. *Mol Microbiol* **36**: 962-972.

Sonden, B., and Uhlin, B.E. (1996) Coordinated and differential expression of histone-like proteins in *Escherichia coli*: regulation and function of the H-NS analog StpA. *EMBO J* **15**: 4970-4980.

Spassky, A., Rimsky, S., Garreau, H., and Buc, H. (1984) H1a, an *E. coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA *in vitro*. *Nucleic Acids Res* **12**: 5321-5340.

Spurio, R., Falconi, M., Brandi, A., Pon, C.L., and Gualerzi, C.O. (1997) The oligomeric structure of nucleoid protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending. *EMBO J* **16**: 1795-1805.

Stallions, D.R., and Curtiss, R., 3rd (1972) Bacterial conjugation under anaerobic conditions. *J Bacteriol* **111**: 294-295.

Starcic-Erjavec, M., van Putten, J.P., Gaastra, W., Jordi, B.J., Grabnar, M., and Zgur-Bertok, D. (2003) H-NS and Lrp serve as positive modulators of *traJ* expression from the *Escherichia coli* plasmid pRK100. *Mol Genet Genomics* **270**: 94-102.

Starcic Erjavec, M., Gaastra, W., van Putten, J., and Zgur-Bertok, D. (2003) Identification of the origin of replications and partial characterization of plasmid pRK100. *Plasmid* **50**: 102-112.

Starcic, M., Zgur-Bertok, D., Jordi, B.J., Wosten, M.M., Gaastra, W., and van Putten, J.P. (2003) The cyclic AMP-cyclic AMP receptor protein complex regulates activity of the *traJ* promoter of the *Escherichia coli* conjugative plasmid pRK100. *J Bacteriol* **185**: 1616-1623.

Steck, T.R., Franco, R.J., Wang, J.Y., and Drlica, K. (1993) Topoisomerase mutations affect the relative abundance of many *Escherichia coli* proteins. *Mol Microbiol* **10**: 473-481.

Stella, S., Spurio, R., Falconi, M., Pon, C.L., and Gualerzi, C.O. (2005) Nature and mechanism of the *in vivo* oligomerization of nucleoid protein H-NS. *Embo J* **24**: 2896-2905.

Stockwell, D., Lelianova, V., Thompson, T., and Dempsey, W.B. (2000) Transcription of the transfer genes *traY* and *traM* of the antibiotic resistance plasmid R100-1 is linked. *Plasmid* **43**: 35-48.

Strohmaier, H., Noiges, R., Kotschan, S., Sawers, G., Hogenauer, G., Zechner, E.L., and Koraimann, G. (1998) Signal transduction and bacterial conjugation: characterization of the role of ArcA in regulating conjugative transfer of the resistance plasmid R1. *J Mol Biol* **277**: 309-316.

Swinger, K.K., Lemberg, K.M., Zhang, Y., and Rice, P.A. (2003) Flexible DNA bending in HU-DNA cocrystal structures. *EMBO J* **22**: 3749-3760.

Taki, K., Abo, T., and Ohtsubo, E. (1998) Regulatory mechanisms in expression of the *traY-I* operon of sex factor plasmid R100: involvement of *traJ* and *traY* gene products. *Genes Cells* **3**: 331-345.

Tobe, T., Yoshikawa, M., Mizuno, T., and Sasakawa, C. (1993) Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by *virF* and repression by H-NS. *J Bacteriol* **175**: 6142-6149.

Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F., and de Laat, W. (2002) Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* **10**: 1453-1465.

Tsai, M.M., Fu, Y.H., and Deonier, R.C. (1990) Intrinsic bends and integration host factor binding at F plasmid *oriT*. *J Bacteriol* **172**: 4603-4609.

Tsui, H.C., Leung, H.C., and Winkler, M.E. (1994) Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. *Mol Microbiol* **13**: 35-49.

Tsui, H.C., Feng, G., and Winkler, M.E. (1997) Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12. *J Bacteriol* **179**: 7476-7487.

Tupper, A.E., Owen-Hughes, T.A., Ussery, D.W., Santos, D.S., Ferguson, D.J., Sidebotham, J.M., Hinton, J.C., and Higgins, C.F. (1994) The chromatin-associated protein H-NS alters DNA topology *in vitro*. *Embo J* **13**: 258-268.

Valentin-Hansen, P., Eriksen, M., and Udesen, C. (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**: 1525-1533.

van Biesen, T., Soderbom, F., Wagner, E.G., and Frost, L.S. (1993) Structural and functional analyses of the FinP antisense RNA regulatory system of the F conjugative plasmid. *Mol Microbiol* **10**: 35-43.

van Biesen, T., and Frost, L.S. (1994) The FinO protein of IncF plasmids binds FinP antisense RNA and its target, *traJ* mRNA, and promotes duplex formation. *Mol Microbiol* **14**: 427-436.

van Workum, M., van Dooren, S.J., Oldenburg, N., Molenaar, D., Jensen, P.R., Snoep, J.L., and Westerhoff, H.V. (1996) DNA supercoiling depends on the phosphorylation potential in *Escherichia coli*. *Mol Microbiol* **20**: 351-360.

Vecerek, B., Moll, I., and Blasi, U. (2005) Translational autocontrol of the *Escherichia coli hfq* RNA chaperone gene. *RNA* **11**: 976-984.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**: 672-676.

Vytvytska, O., Jakobsen, J.S., Balcunaite, G., Andersen, J.S., Baccarini, M., and von Gabain, A. (1998) Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability. *Proc Natl Acad Sci USA* **95**: 14118-14123.

Vytvytska, O., Moll, I., Kaberdin, V.R., von Gabain, A., and Blasi, U. (2000) Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding. *Genes Dev* **14**: 1109-1118.

Walker, K.A., Atkins, C.L., and Osuna, R. (1999) Functional determinants of the *Escherichia coli fis* promoter: roles of -35, -10, and transcription initiation regions in the response to stringent control and growth phase-dependent regulation. *J Bacteriol* **181**: 1269-1280.

Walker, K.A., and Osuna, R. (2002) Factors affecting start site selection at the *Escherichia coli fis* promoter. *J Bacteriol* **184**: 4783-4791.

Walker, K.A., Mallik, P., Pratt, T.S., and Osuna, R. (2004) The *Escherichia coli* Fis promoter is regulated by changes in the levels of its transcription initiation nucleotide CTP. *J Biol Chem* **279**: 50818-50828.

Wang, J.Y., and Syvanen, M. (1992) DNA twist as a transcriptional sensor for environmental changes. *Mol Microbiol* **6**: 1861-1866.

Wanner, B.L. (1993) Gene regulation by phosphate in enteric bacteria. *J Cell Biochem* **51**: 47-54.

White, J.H. (1969) Self-Linking and the Gauss Integral in Higher Dimensions. *Am J Math* **91**: 693-728.

Will, W.R., Lu, J., and Frost, L.S. (2004) The role of H-NS in silencing F transfer gene expression during entry into stationary phase. *Mol Microbiol* **54**: 769-782.

Willetts, N. (1977) The transcriptional control of fertility in F-like plasmids. *J Mol Biol* **112**: 141-148.

Williams, R.M., and Rimsky, S. (1997) Molecular aspects of the *E. coli* nucleoid protein, H-NS: a central controller of gene regulatory networks. *FEMS Microbiol Lett* **156**: 175-185.

Wilson, R.L., Libby, S.J., Freet, A.M., Boddicker, J.D., Fahlen, T.F., and Jones, B.D. (2001) Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. *Mol Microbiol* **39**: 79-88.

Wolfe, A.J., Chang, D.E., Walker, J.D., Seitz-Partridge, J.E., Vidaurri, M.D., Lange, C.F., Pruss, B.M., Henk, M.C., Larkin, J.C., and Conway, T. (2003) Evidence that acetyl phosphate functions as a global signal during biofilm development. *Mol Microbiol* **48**: 977-988.

Wolfe, A.J. (2005) The acetate switch. *Microbiol Mol Biol Rev* **69**: 12-50.

Wu, H.Y., Shyy, S.H., Wang, J.C., and Liu, L.F. (1988) Transcription generates positively and negatively supercoiled domains in the template. *Cell* **53**: 433-440.

Zechiedrich, E.L., Khodursky, A.B., Bachellier, S., Schneider, R., Chen, D., Lilley, D.M., and Cozzarelli, N.R. (2000) Roles of topoisomerases in maintaining steady-state DNA supercoiling in *Escherichia coli*. *J Biol Chem* **275**: 8103-8113.

Zechner, E.L., De la Cruz, F., Eisenbrandt, R., Grahn, A.M., Koraimann, G., Lanka, E., Muth, G., Pansegrau, W., Thomas, C.M., Wilkins, B.M., and Zatyka, M. (2000) Conjugative DNA-Transfer Processes. In *The Horizontal Gene Pool*. Thomas, C.M. (ed). Amsterdam: Harwood Academic Publishers, pp. 87-174.

Zhang, A., and Belfort, M. (1992) Nucleotide sequence of a newly-identified *Escherichia coli* gene, *stpA*, encoding an H-NS-like protein. *Nucleic Acids Res* **20**: 6735.

Zhang, A., Derbyshire, V., Salvo, J.L., and Belfort, M. (1995) *Escherichia coli* protein StpA stimulates self-splicing by promoting RNA assembly *in vitro*. *RNA* **1**: 783-793.

Zhang, A., Rimsky, S., Reaban, M.E., Buc, H., and Belfort, M. (1996) *Escherichia coli* protein analogs StpA and H-NS: regulatory loops, similar and disparate effects on nucleic acid dynamics. *EMBO J* **15**: 1340-1349.

Zhang, A., Wassarman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **9**: 11-22.

Zhang, X., and Bremer, H. (1996) Effects of Fis on ribosome synthesis and activity and on rRNA promoter activities in *Escherichia coli*. *J Mol Biol* **259**: 27-40.

Zuber, F., Kotlarz, D., Rimsky, S., and Buc, H. (1994) Modulated expression of promoters containing upstream curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. *Mol Microbiol* **12**: 231-240.